PHD

The synthesis of novel anticancer agents with therapeutic potential

Ganeshapillai, Dharshini

Award date:
2001

Awarding institution:
University of Bath

THE SYNTHESIS OF NOVEL ANTICANCER AGENTS WITH THERAPEUTIC POTENTIAL

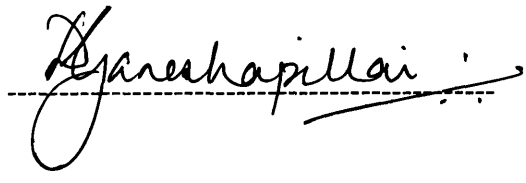
Submitted by

Dharshini Ganeshapillai

for the degree of Doctor of Philosophy

of the University of Bath

2001



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For the loving memory of my dad
and to my mum for her endless love and devotion

Abstract

Inhibition of steroid sulphatase activity is an important target for the development of new drugs for oncology and immunology. In this work, synthetic procedures to synthesise novel, potent non-steroidal, non-estrogenic sulphamate derivatives for the treatment of postmenopausal women with hormone dependent breast cancer, and 2-substituted estrone-3-sulphamide, 3-sulphonamide and 3-*S*-sulphamate derivatives as potential antitubulin, antiangiogenic and apoptotic inducers are described.

The strategies adopted to develop STS inhibitors are to develop compounds which are active *in vivo* and devoid of any estrogenicity, an unwanted property that would limit their clinical use such as the potent estrone-3-*O*-sulphamate (EMATE). Structure-activity studies were carried out on the lead 4-methylcoumarin-7-*O*-sulphamate (COUMATE) by incorporating longer alkyl chains and other functionalities at the C-4 and/or C-3 positions of the coumarin ring. Also, a fused third ring was incorporated to give tricyclic coumarin sulphamates, which structurally might mimic the ABCD rings of the steroid. Similar to coumarin sulphamates other heterocyclic ring containing sulphamates are also of considerable interest. Therefore, several novel 1, 2, and/or 3-substituted indole sulphamates were also syntheisesd.

The best inhibitors in the C-3 and C-4 alkyl series were those with 7-9 and 6-9 carbon-containing alkyl chains at the C-3 or C-4 positions, respectively inhibiting the E1-STS activity in MCF-7 cells at 0.01 μM by 85-91% and >90%. (c.f. 35% for COUMATE). The 3-benzyl-4-methylcoumarin-7-*O*-sulphamate **(95)** was found to be a potent inhibitor with an IC_{50} of 8 nM. In the tricyclic coumarin-based analogues with a third ring of sizes between 5 and 15 carbons (665-6615 COUMATES), more powerful active site-directed inhibitors than EMATE and its analogues were found. The most potent compounds in this series, 669 COUMATE **(119)** and 6610 COUMATE **(122)** have IC_{50}'s of 2.4 and 1 nM, respectively *in vitro* and are non-estrogenic *in vivo*. Interestingly, 6615 COUMATE **(131)** proved to be the most active congener *in vivo*. The higher potencies observed for the alkyl series of compounds can be due to their bicyclic system mimicking the A/B rings of steroid backbone with their C-3 or C-4 alkyl substituents interacting with the amino acid residues at the active site, which normally recognise the C/D rings of steroid. The higher inhibition observed for the tricyclic COUMATEs could be attributed to the fact that the third cyclic rings might fold in such a way to mimic the C/D ring conformation of the steroid and hence have better recognition for binding at the enzyme active site. In addition, the lipophilicity of the tricyclic compounds was studied by calculating the log P values by an HPLC technique and the X-ray crystal structures of compounds **119** and **122** were determined.

2-Methoxyestradiol, a natural estradiol metabolite, is non-estrogenic, known to induce G2/M phase cell cycle arrest and apoptosis. The sulphamate derivatives of this compound, 2-MeO/2-EthylEMATE and 2-MeO/2-EtE2MATE have previously been found to be more potent, irreversible and non-estrogenic inhibitors, and also shown to trigger apoptosis and inhibit tubulin polymerisation. It is therefore possible that the structurally similar derivatives of these synthetic sulphamates, such as the 2-substituted

3-sulphamide, 3-sulphonamide and 3-*S*-sulphamate, with nitrogen or sulphur bridging heteroatoms at the C-3 position would also inhibit tubulin polymerisation and angiogenesis. These compounds would provide an indication about the importance of the sulphamoyloxy group for potent activity in 2-MeOEMATE, which is thought to elicit effect by taken up into blood cells and released slowly. Therefore, presumably acting as a pro drug of 2-MeOE1. There are certain sulphonamides, which are known to bind to carbonic anhydrase in the blood cells therefore the sulphonamides syntheised might also show similar inhibitory activities to that of the sulphamates. Most importantly, these compounds would offer useful information about the site where the sulphamoyl group binds in tubulin and the mechanism of binding for irreversible inhibition, which might be similar to that of sulphamoylation of sulphatase enzyme. This structure-activity study would also show how the stability of the sulphamoyloxy group could be improved further. Therefore, novel sulphamate surrogates such as 2-methoxy and 2-ethyl 3-sulphamide, 3-sulphonamide and 3-*S*-sulphamates are synthesised in this work and their synthetic methods are reported.

Acknowledgements

I would like to express my gratitude to my supervisor, Professor B. V. L. Potter for giving me an opportunity to work on this project and for his inspiring guidance and enthusiasm through out this project.

My heartfelt thanks to Dr. Lawrence Woo for all the encouragement and his patience in reading my thesis. My sincere thanks to Dr. Mike Threadgill for his generous support and helpful advice throughout the course of this work.

I wish to extend my appreciation to Professor Mike Reed and Dr. Atul Purohit at the Endocrinology and Metabolic Medicine, Imperial College School of Medicine, St. Mary's Hospital, London for the biological evaluation of all my compounds, for their helpful suggestion and friendship. My sincere thanks to Dr. Mary Mahon at the Department of Chemistry, University of Bath, for X-ray crystallography and Mr. Kevin Smith for molecular modelling of my compounds. Special thanks are also to all the technical staff at the University of Bath for NMR, mass spectral and microanalysis services.

I would like to thank all my friends in the department, especially Hatem, Ifat and Jackie for their friendship and encouragement and for all the fun. My thanks are also due to Sarah Al-Benna and Shabhano Ali for being good friends and for their moral support.

Finally, I am indebted to my mum for all her love, encouragement and endless support throughout my life and she is the best mum in the world. I would like to thank Suthan for his love, encouragement and being very understanding, tolerant and supportive all the way. My sincere thanks to my sisters, family and friends for their encouragement and support.

Publications

Some of the work described in this thesis have been published in the following publications or in preparation:

B. Malini, A. Purohit, D. Ganeshapillai, L. W. L. Woo, B. V. L. Potter, M. J. Reed. Inhibition of steroid sulphatase activity by tricyclic coumarin sulphamates; *Journal of Steroid Biochemistry and Molecular Biology*, **2000**, 75, 4-5, 253-258.

D. Ganeshapillai, L. W. L. Woo, M. J. Reed, B. V. L. Potter. Tricyclic coumarin sulphamates : potent, non-steroidal inhibitors of estrone sulphatase; (2001), *Manuscript in preparation.*

D. Ganeshapillai, L. W. L. Woo, M. J. Reed, B. V. L. Potter. Substituted coumarin sulphamates : potent, non-steroidal inhibitors of estrone sulphatase; (2001), *Manuscript in preparation.*

D. Ganeshapillai, L. W. L. Woo, M. F. Mahon, M. J. Reed, B. V. L. Potter. X-ray crystal structural analysis of the tricyclic coumarin sulphamates; (2001), *Manuscript in preparation.*

L. Wood, M. P. Leese, B. Leblond, L. W. L. Woo, D. Ganeshapillai, A. Purohit, M. J. Reed, B. V. L. Potter, G. Packham; Inhibition of Superoxide Dismutase by 2-Methoxyestradiol analogues and estrogen derivatives: Structure/activity relationships. (2001), *Manuscript submitted.*

Abbreviations

ACTH	Adrenocorticotropic hormone
ADME	Absorption, distribution, metabolism and excretion
Ac	Acetyl
Adiol	Androstenediol
AG	Aminoglutethimide
Anhy.	Anhydrous
AR	Aromatase
ASA	Arylsulphatase A
ASB	Arylsulphatase B
ATP	Adenosine trisphosphate
aq.	Aqueous
Bn	Benzyl (CH_2Ph)
br.	Broad (spectra)
bp	Boiling Point
Bz	Benzoyl (COPh)
δ	Chemical shift (spectra)
ca	Approximately
COX-2	Cyclooxygenase-2
cm	Centimeter(s)
conc.	Concentrated
CDK	Cyclin dependent kinase
CNS	Central nervous system
COMT	Catachol-*O*-methyltransferase
COUMATE	4-Methylcoumarin-7-*O*-sulphamate
CREB	cAMP response element binding protein
cAMP	Cyclic adenosine monophosphate
Cys	Cysteine
°C	Degree Celsius
Δ	Symbol for heat supplied to a reaction
d	Doublet (spectra)
dd	Doublet of doublet (spectra)
DES	Diethylstilbestrol
DHA	Dehydroepiandrosterone
DHA-S	Dehydroepiandrosterone sulphate
DHA-STS	Dehydroepiandrosterone sulphatase
DMA	*N,N*-Dimethylacetamide
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
E	Cortisone
E1	Estrone
E1-MTP	Estrone-3-*O*-methylthiophosphonate
EMATE	Estrone-3-*O*-sulphamate
E1S	Estrone sulphate
E1-ST	Estrone sulphotransferase
E1-STS	Estrone sulphatase
E2	Estradiol
E3	Estriol
e.i.	Electron ionisation
ER	Estrogen receptor
ER^+	Estrogen receptor-positive
ER^-	Estrogen receptor-negative
Er	Endoplasmic reticulum
Et_2O	Diethyl ether
F	Cortosol
FAB	Fast atom bombardment
FGly	Formylglycine
FSH	Follicle-stimulating hormone
GAL6S	*N*-acetylgalactosamine-6-sulphatase
GLU6S	*N*-acetylglucosamine-6-sulphatase
HDBC	Hormone dependent breast cancer
hER	Human estrogen receptor
HIV	Human Immuno-deficiency Virus
HRT	Hormone replacement therapy
17β-HSD	17β-hydroxysteroid dehydrogenase
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IR	Infra-red
^iPr	isopropyl
J	Coupling constant

LDA	Lithium diisopropylamide
LH	Luteinising hormone
m	Multiplet
mp	Melting Point
2-MeOE2	2-Methoxyestradiol
MHz	Megahertz
MLD	Metachromatic eukodystrophy
***m*-NBA**	*m*-Nitrobenzyl alcohol
mRNA	Messenger Ribonuceic Acid
MS	Mass Spectra
MSD	Multiple sulphatase deficiency
MSP	Mucopolysaccharidosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NHDBC	Non-hormone dependent breast cancer
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drugs
P_{450}	Cytochrome P_{450} enzyme
PDA	Photo Diode Array
PMA	Phosphomolybic acid
PR	Progestrone Receptor
PR^+	Progestrone Receptor positive
q	Quartet (spectra)
R_f	Retention factor
RBC	Red blood cells
RNA	Ribonucleic Acid
R.T.	Room temperature
s	Singlet (spectra)
4-S	Arylsulphatase B
SD	Standard Deviation
SF-1	Steroidogenic factor-1
STS	Steroid sulphatase
SERM	Selective Estrogen Receptor Modulator
t	Triplet (spectra)
t_R	Retention time
^tBu	Tertiary butyl
Bu_4NCl	Tertabutylammonium chloride
Bu_4NI	Tertabutylammonium iodide
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
THN	Tetrahydronaphth-2-ol
TLC	Thin layer chromatography
VEGF	Vascular endothelial growth factor
UV	Ultra Violet

Abbreviations for the steroid compounds used throughout the text

Trival Name	Approved Name
Androstenedione	4-Androstene-3,17-dione
Cholesterol	5-Cholesten-3β-ol
Dehydroepiandrostenedione	5-Androsten-3β-ol-17-one
Estradiol	1,3,5(10)-Estratriene-3-17β-diol
Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol
Estrone	3-Hydroxy-1,3,5(10)-estratriene-3-one
Progesterone	4-Pregnene-3,20-dione
Pregnenolone	3β-Hydroxy-5-pregnen-20-one
Testosterone	17β-Hydroxy-4-androsten-3-one

The nomenclature of organic compounds adopted in this thesis follows the rule of International Union of Pure and Applied Chemistry (IUPAC).

The references in the text are cited by means of subscript numbers, which are run seriatim through out the thesis.

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CHAPTER 1

1.0 Steroids

Steroids are derived from cholesterol, so they are highly lipophilic and readily enter most cells to access their intracellular receptors. Many of the important steroids, such as cholesterol, cholic acid and ergosterol were first isolated in the 19th century, but were not characterised until the beginning of the 20th century. The early structural work was carried out in 1932 in Germany by Adolf Windaus and Heinrich Wieland, followed by Otto Rosenheim and Harold King of the National Institute of Medical Research in England, for which they received the most prestigious Nobel Prize.[1]

Estrone (E1) was the first sex hormone to be recognised in 1929 by Edward Doisy in USA, followed by pregnanediol and estriol (E3) by G. F. Marion in 1930, androsterone by Adolf Butenandt in 1932 and testosterone by Ernst Laqueur in 1935. At the beginning of World War II, cortisone (E) was synthesised by Lewis Sarett in USA and found to have a remarkable effect in the treatment of rheumatoid arthritis.[1] This discovery set off a trend to synthesise steroidal and non-steroidal anti-inflammatories.

In 1932, Robert Robinson first attempted to propose routes for the synthesis of steroids, which later served as a reference point for the total synthesis of estrogens, stilbestrol and still later potent tumour-inhibiting compounds such as tamoxifen.[2] Since then, R. B. Woodward (total synthesis of Androgens), K. Ziegler (7-dehydrocholesterol), D. H. R. Barton, E. R. H. Jones (ergosterol), B. Henbest and several others revealed elegant and historic synthetic methods for androgenic hormones and their derivatives.[2]

The conformational analysis and the elucidation of steroid stereochemistry by Sir Derek Barton in 1950 created a new turn in the fundamental investigation of steroids. As a consequence of this ongoing worldwide race, various other important steroids were isolated, characterised and synthesised. The studies on steroids have become an internationally renowned area of research ever since.[1,2]

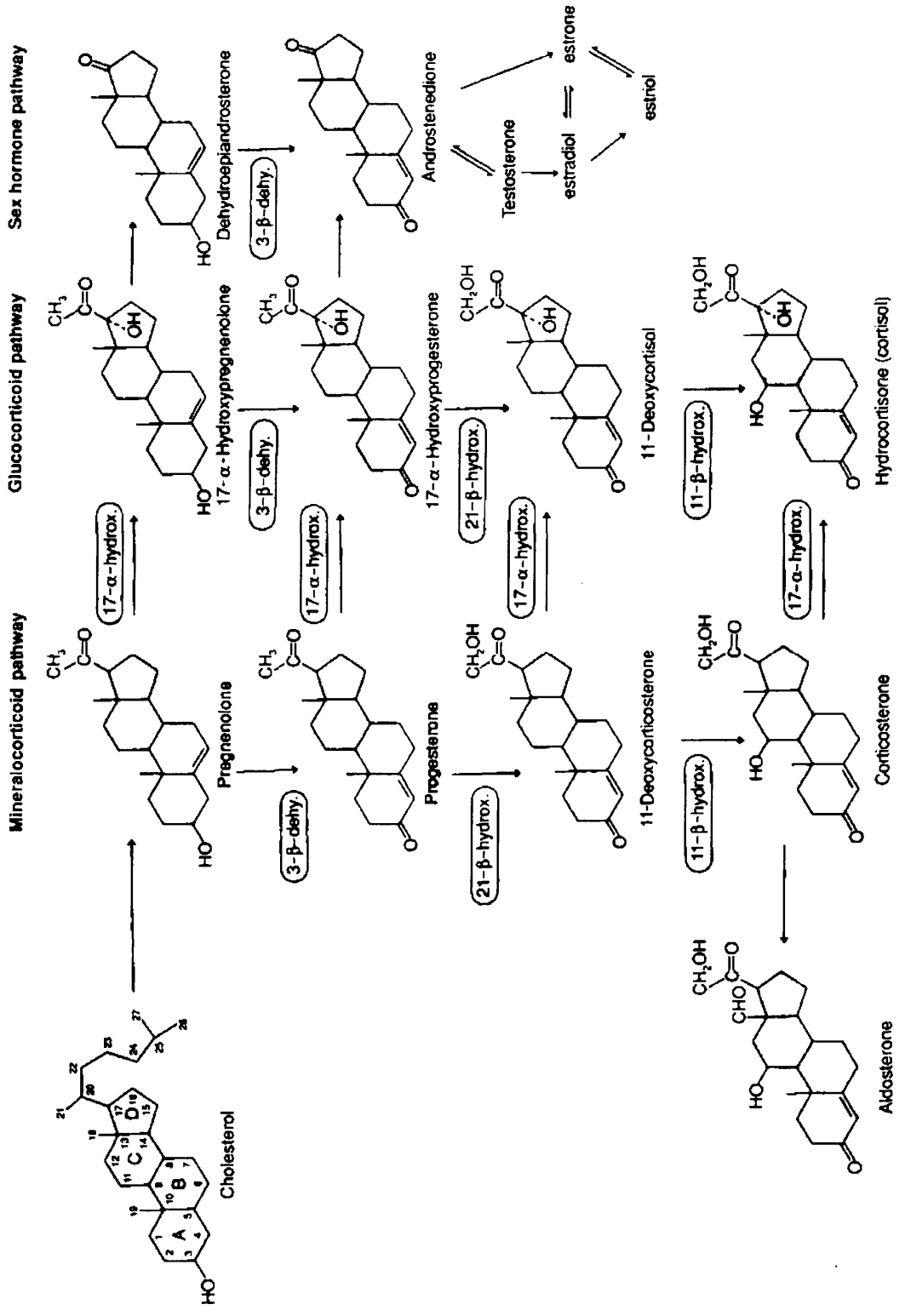


Figure 1.1 : Biosynthesis of steroid hormones[3]

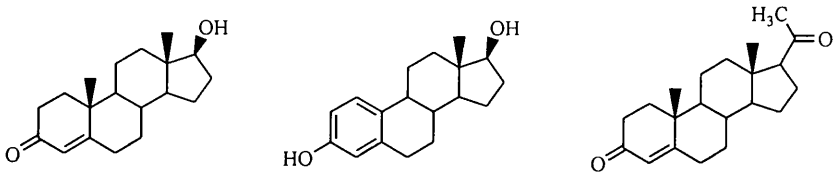
(Hydrox. : Hydroxy steroid dehydrogenase; Dehy. : Dehydrogenase)

1.1 Biosynthesis of steroid hormones

The biosynthetic pathway of steroids is very complex since they are derived from a single precursor, cholesterol. Cholesterol, besides being ingested in food, is synthesised in large amounts in the body. An adult human contains about 250 g of cholesterol although the steroids from which they are produced are at milligram level or lower.[3] The origin of all the compounds involved in steroid synthesis are acetate, in the form of acetyl co-enzyme A.[3] In addition, the presence of oxygen, the reduced form of nicotinamide adinine dinucleotide phosphate (NADPH) and other co-factors are also required for the synthesis.[3,4]

Steroid hormones are synthesised by steroidogenic tissue of adrenal and gonads from cholesterol within the elements of the endoplasmic reticulum (Er). Steroid-secreting cells are easily identified by the large amounts of smooth Er present in these cells. The complex multiple enzyme system required for the synthesis of steroids is present within mitochondria as well as in the cytoplasm.

The adrenal steroidogenic tissue produces glucocorticoids such as cortisol (F), cortisone (E) and mineralocorticoids such as aldosterone[4] (Figure 1.1). The steroidogenic tissue of the gonads produces a number of sex steroids such as androgens (masculinising), estrogens (feminising) and progestins (related to pregnancy). The testes produce testosterone **(1-1)** and the ovaries produce estradiol (E2) **(1-2)** and progestrone **(1-3)** (Figure 1.2). During pregnancy, the placenta is an additional source of estrogens and progestins.[4]



Testosterone **1-1** **Estradiol** **1-2** **Progestrone** **1-3**

Figure 1.2 : Structures of steroid hormones from the gonads

The regulation of steroid biosynthesis is achieved by an intricate network of peptide hormones, which are under neuro-endocrine influence, starting from the hypothalamus in the central nervous system (CNS). The hypothalamus produces a number of small peptide hormones that act as releasing factors for the hormones synthesised from the pituitary gland. Adrenocorticotropic hormone (ACTH) regulates corticosteroid synthesis in the adrenal cortex, whereas luteinising hormone (LH) and follicle-stimulating hormone (FSH) act on the ovaries and testes. Gonodotropins induce the production of estrogens and gestagens in the female. In the male, LH and FSH regulate androgen formation. At the same time, the steroids have a feed back regulatory effect on the hypothalamo-pituitary axis, setting up an exquisitely tuned regulatory loop.[4,3]

Even though, many hormones are stored in the cells as granules, steroid hormones are not stored, to any extent, in vesicles. Being lipophilic and soluble in lipids, they are carried in plasma by carrier molecules and hence readily cross the plasma membrane of the cell. Therefore, their rate of release is directly related to their rate of synthesis. Steroids are bound to various plasma proteins once released into the blood stream.[3] The free form is usually only a small fraction of the total hormone in the blood. There is equilibrium between the bound and free form, but only the free form can penetrate the cell membrane and elicit an effect in the target cells. The concentration of the active hormone in the blood is therefore, determined by the dynamic relationship between its rate of secretion, the degree to which it is bound to plasma proteins and its rate of inactivation.[4]

Hormones in general affect the growth, development, metabolic activity and function of tissues. The responses are often the result of a combination of the action of several hormones. These actions may be stimulatory or inhibitory and additive or synergistic. Once inside the cell, the first step of a steroid hormone is to bind to its specific receptor, which can be a monomeric or dimeric accepting one or two molecules of steroid hormones.[5] The steroid-receptor complex is then translocates to the nucleus where it acts on a particular segment of the chromosomal DNA. This results in an induction of gene activation, which leads to transcription of new messenger RNA

species and consequently specific effector protein synthesis rendering cellular effect in the target cell[6] (Figure 1.3).

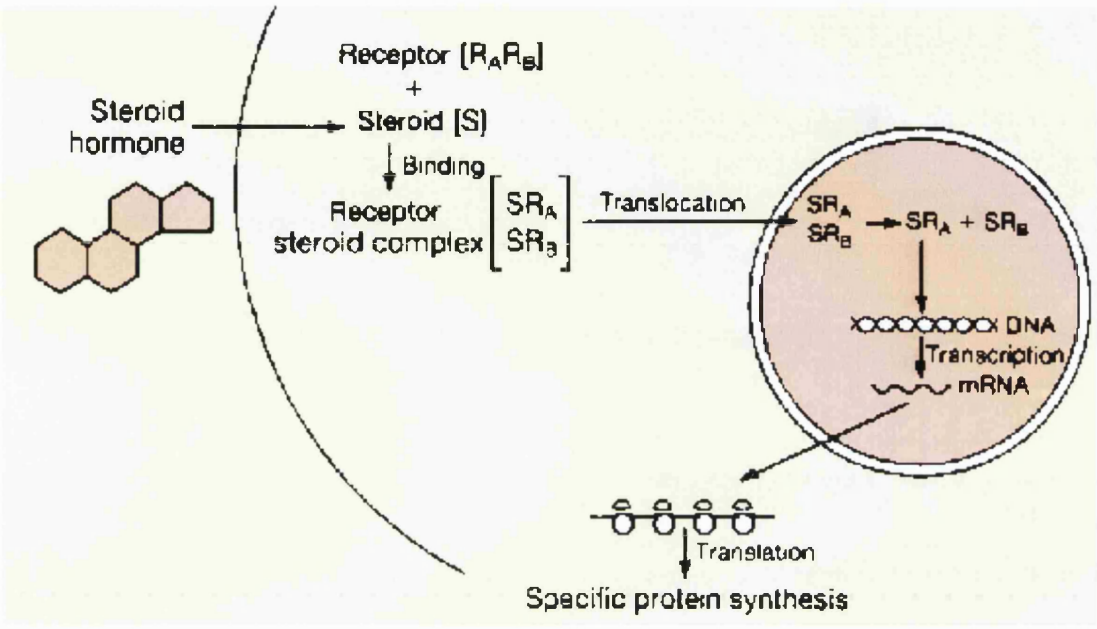
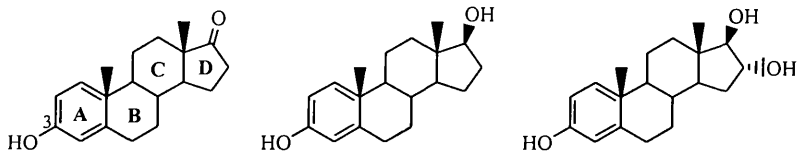


Figure 1.3 : Steroid hormone action at the cellular level.[6]

Inactivation of hormones may occur in the blood, liver, the kidneys, and in some cases the target tissues itself, by degradation, oxidation, reduction or methylation. The metabolites so formed often conjugate with glucuronic acid and are excreted in the urine or bile.[3] The body's endocrine system acts very slowly since it takes time for hormones to be synthesised, released and transported *via* the blood stream to some distant target organ. The concentrations of hormones in the blood are extremely low in the range of 10^{-6} to 10^{-9} mol/l.[3] Although the onset is rather slow, the hormonal effects are longer acting than those of the neurones from a matter of minutes to days. Hormones do not disappear from the body rapidly, nor do the intracellular effects turn off immediately as do the termination of neuronal stimulation when the action potentials no longer reach their effector organ.[4] This makes the whole endocrine system the most important and by far the most complex in the body.

1.2 Estrogens and their biosynthesis

Estrogens are the most important factors for the growth and maintenance of the breast, vagina and uterus and also the development of the secondary sexual characteristics in women.[7] Estrogens are characterised by an aromatic ring A with a phenolic hydroxyl group at the C-3 position. It is the unique nature of the A ring which distinguishes its chemistry from other steroids. About 20 different biologically active estrogens have been identified, but estrone (E1) **(1-4)**, estradiol (E2) **(1-2)** and estriol (E3) **(1-5)** are the three principal ones of clinical significance[7] (Figure1.4).



Estrone (E1) **1-4** | **Estradiol (E2)** **1-2** | **Estriol (E3)** **1-5**

Figure 1.4 : The natural estrogens

Out of these three estrogens, E2 is the most biologically active and abundant in tissues, responsible for the onset of heat in animals and the control of menstrual cycle in women. In the liver, E2 is readily oxidised to E1 by 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme, which in turn can be converted to E3 in the liver itself.[7] The metabolites of these estrogens (particularly E3 and pregnanediol) are excreted by the kidney. E1 and E2 exist in equilibrium at a ratio of 1:2 to 1:4. E3 is an estrogen of low estragenicity and has been regarded as only a partial agonist. However, the true situation is that E3 is short acting but if its concentration in the plasma is maintained under physiological conditions, E3 can be as potent as E2.

In premenopausal women, estrogens are produced predominantly in the granulosa cells of the ovarian follicles from androgens by the stimulation of FSH. After ovulation it is produced by the corpus luteum.[8] The production of estrogens is stimulated during the first and second half of the menstrual cycle, where LH combines with FSH in the second half.[8] Upon binding of FSH to its G-protein coupled receptor in the granulosa

cell membrane, the intracellular cyclic adenosine monophosphate (cAMP) levels rise and the binding of two critical transcription factors, i.e. steroidogenic factor-1 (SF-1) and cAMP response element binding protein (CREB), to the classically located proximal promoter II of the aromatase (AR) gene is enhanced.[9,10] This, in turn, activates AR expression and consequently causes estrogen production and secretion from the pre-ovulatory follicle.[11]

Estrogens are also synthesised by the placenta in large quantities during pregnancy, in small amounts by the testes in males and by the adrenal cortex in both sexes.[12] Other tissues such as liver,[13] muscle,[14] fat,[14] brain[15] and hair follicles[16] can also convert steroid precursors into estrogens. The immediate precursors for estrogen synthesis are androstenedione or testosterone[17] (Figure 1.1).

In postmenopausal women, when the ovaries cease to produce estrogens, adrenal glands take over the function of the ovaries and become the major source of estrogens. The adrenals do not secrete estrogens directly.[18] They secrete indirectly under the influence of ACTH by a process called cortisol feedback control *via* the aromatisation of androstenedione by the AR enzyme in non-ovarian tissues such as liver, muscle, fat, brain, and hair follicle.[19] In contrast to cAMP regulation of AR expression in the ovary in premenopausal women, the production of estrogens in postmenopausal women is controlled primarily by cytokines (IL6, IL-11, TNFα) and glucocorticoids *via* the alternative use of promotor I.4 in adipose tissue and skin fibroblasts.[11,18] The major substrate for AR in adipose and skin is androstenedione of adrenal origin. In postmenopausal women, approximately 2% of circulating androstenedione is converted to E1, which is subsequently converted to the most biologically active E2 in the extra ovarian tissues by the enzyme 17β-HSD.[20,19]

Apart from the natural estrogens such as E1, E2 and E3, there are several other synthetic estrogens such as ethinylestradiol[21] **(1-6)**, mestranol **(1-7)**, hexestrol **(1-8)**, diethylstilbestrol[22] **(1-9)**, dienestrol **(1-10)** and megestrol **(1-11)**, which are widely available clinically (Figure 1.5). Equilin[23] **(1-12)** (Figure 1.5) is another synthetic

estrogen currently being used in estrogen replacement therapy in the patients whose secretions of natural estrogens are inadequate.

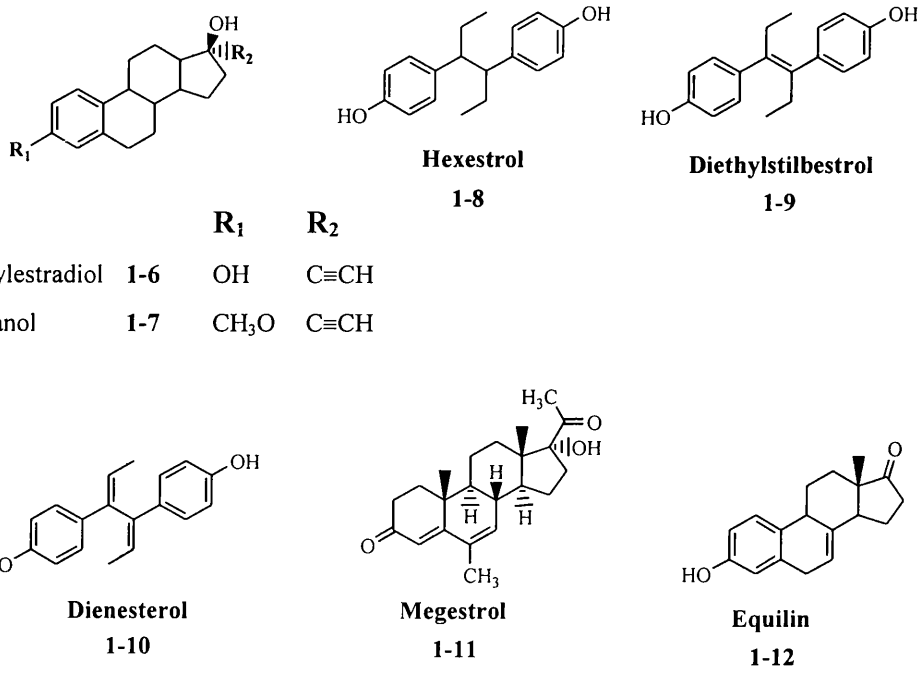


Figure 1.5 : Structures of some widely used synthetic estrogens

Synthetic estrogens are orally administered in children aged between 11 and 13 with primary hypogonadism to stimulate the development of secondary sexual characteristics and for accelerated growth. The main use of synthetic estrogens in adult women is for oral contraception[24] and also for primary amenorrhoea in which case they are used together with a progestogen to induce an artificial cycle. It is also useful to reduce menopausal symptoms associated with the decline in estrogen production, such as hot flushes, inappropriate sweating, palpitations, and atrophic vaginitis. Administration of synthetic estrogens has also been shown to decrease postmenopausal osteoporosis. They maintain the scaffolding of the bone by decreasing the loss of calcium phosphate complexes and protein matrix from the bone.[25,26]

Estrogens that are administered orally as contraceptives have been shown to associate with an elevation in blood coagulation. This is directly related to an increase in platelet

aggregation as a result of an increase in various clotting factor concentrations in the plasma rendering changes in the fibrinolytic system when the antithrombin III concentration is decreased. The coagulability of blood is the basis of increased risk of thromboembolism, associated with contraceptive pills, in particular with those contain a high estrogen content.[24]

1.3 Estrogens and their role in breast cancer

The influence of ovarian estrogens in the development of breast cancer has long been recognised. Animal studies have repeatedly shown that estrogen administration can induce and promote mammary tumours in rodents.[27,28] It is generally believed that estrogens act as promoters rather than as carcinogens in the development of breast tumours. Although, E2 is generally considered to be the major estrogen, which supports tumour growth there is now substantial evidence to show that in postmenopausal women another steroid, 5-androstenediol can also bind to the estrogen receptors (ER) and is equipotent to E2 in its ability to support tumour growth.

In postmenopausal women, in whom breast cancer most frequently occurs, approximately 30-40% of the tumours are hormone dependent.[29] The concentrations of estrogens in particular E2, in breast tumours of these patents are higher than the estrogen levels in normal tissue and blood from the same individual.[30]

1.4 Incidence of breast cancer

Any development of new therapies for the treatment of breast cancer is an important target since the disease is the third most common cancer world-wide and also the most prevalent cancer in women. Breast cancer is the major cause of death in Western women and it ranks third in developed countries and fifth in the developing countries.[31] Breast cancer occurs most frequently in postmenopausal women at a time when the ovaries have ceased to produce estrogens.[32] Incidence rates are high in most industrialised countries, except Japan and low in central and tropical South America, Africa and Asia. In Britain about 40,000 new cases of breast cancer are diagnosed each year and it is now identified to be the most prevalent cancer of all. About 15,000 deaths

are reported each year to be related to this disease, and such death rate is the highest amongst the European community.[33] Therefore, it has been suggested that this international differences in the incidence rate of breast cancer is predominantly due to environmental rather than genetic factors.

The cause of breast cancer, like that of the other cancers, is yet to be fully understood. Research has shown that breast cancer frequently occurs in women aged between 30 and 70 and indicates that reproductive and hormonal factors are the possible causes.[34,35] Breast cancer is found to be less common in women whose first pregnancy occurs before the age of 30.[31] It is also less common in women with a late menarche (>12 years) and who have a regular menses.[36] A reduction in risk is also associated with women who are multiparous, whose ovaries are removed before the age of 37, who breast-fed their babies,[37] who have an early menopause (< 45 years)[38] and who are from the lower socio-economic group and of non-Caucasian race.[39,40]

Factors that cause an increase risk of breast cancer include, obesity in postmenopausal women,[41] a prolonged use of oral contraceptives[42,24] or hormone replacement therapy (HRT),[43] high consumption of unsaturated fats[44] and alcohol[45] and an exposure to high energy radiation.[46] In addition, women who have blood relatives (mother or sisters) affected by this disease[47] and who have a previous history of other cystic diseases, such as chronic mastitis and who have been treated with androgens have an increase risk of developing breast cancer at any one point of their life time.[48] Epidemiological studies have shown that breast cancer is a heterogeneous disease with all women in general are randomly at risk of developing it at some stage of their lives, but data are not available to suggest any alternatives to avoid any risk factors.

1.5 Estrogen and progestrone receptors

In the past decade, the discovery of the estrogen receptors (ER) within the hormone dependent primary breast tumour cells and the development of effective, but relatively non-toxic hormonal drugs have revolutionised the treatment and understanding of breast cancer. The inability in the past to identify potential responders to endocrine

therapy prior to treatment had offered a large proportion of breast cancer patients no alternative but invasive procedures. In seeking to improve the effectiveness of treatment as well as patient compliance, patients are now screened for their ER status and categorised according to the hormone dependency of their breast tumours.

Evidence to support the presence of ER within the target tissues was first shown in the early 1960's. In order for estrogens to regulate cell growth and other functions in humans, a specific protein called the human estrogen receptor (hER) must be present.[49] Much of the hER are initially found in the cytoplasm but, now it has been shown that these receptors are predominantly present in the nucleus and the initial observation was due to liberation of free receptors from the nucleus during homogenisation.[50]

The hER gene has been cloned, sequenced and expressed and it was found to be composed of two non-overlapping functional domains, which have higher affinity for estrogens and DNA respectively.[51] When estrogen binds to the first domain, a conformational change is triggered in the second domain, which allows the interaction with the specific DNA nucleotides known as a transcriptional enhancer region. As a result, an increased production of mRNA from selected genes that are essential for tumour growth occurs.[52]

Patients are classified as estrogen-receptor positive (ER^+) or estrogen-receptor negative (ER^-) according to the concentration of ER protein in the breast tissue. One with higher concentration of receptor protein than the average of 10 fmol receptor protein/mg breast tissue is considered to be ER^+ and one with less than this value is regarded as ER^-.[53] Analysis of primary tumours has shown that 50-60% of all tumours contains measurable quantities of ER^+ tumours.[54] Patients whose tumours expressed little or no ER rarely responded to endocrine therapy, whereas, 60% of patients with ER^+ tumours responded. The remaining third of patients with ER^+ tumours that failed to respond to hormone manipulation may not contain a fully functional ER system. 60% of tumours in premenopausal women are ER^+ and this rises to 80% in postmenopausal women. The frequency of ER^+ tumours in postmenopausal woman was found to be

significantly higher than in premenopausal women. Age rather than menopausal status was found to be associated with this difference.[55] The majority of patients with ER^+ tumours and about 10% of ER^- tumours respond to most types of endocrine therapy.

In addition to ER, the presence of progesterone receptors (PR) has been identified in breast tumours. The frequency of progesterone receptor positive (PR^+) tumours was found to be significantly lower in postmenopausal woman than in the premenopausal women.[55] However, unlike the situation in ER tumours, neither age nor the menopausal status alone could account for such difference but it appears to be due to a combined effect of the two factors. The role of PR in breast cancer is yet to be fully understood but it is possible that PR acts as a mediator of hormonal response. The proliferation of tumour cells induced by estrogen is thought to be due to the presence of both ER and PR.[56] It has been identified that PR is present in the majority of ER^+ patients, but to a lesser extent in ER^- patients. Patients who are PR^+ and ER^+ were found to respond to endocrine therapy much better (73%) than patients with other combinations of ER and PR status.[57] Therefore, the ER and PR status of a breast tumour is a useful tool for predicting the response of the patient to the endocrine therapy administered.[58]

1.6 Primary treatment of breast cancer

Breast cancers can be broadly divided into two types: Hormone dependent breast cancer (HDBC) and non-hormone dependent breast cancer (NHDBC). In post menopausal women, about 1/3 of the patients have hormone dependent breast tumour whose growth and development are supported by estrogens derived from extraglandular tissues such as the adipose, muscle, normal and malignant breast tissues.[33]

Breast cancer is one of only a few cancers (cancers of the cervix, breast and mouth) whose incidence and prognosis could be altered by both primary and secondary preventive measures, which are practical enough to be implemented. In primary prevention, reproductive modification is not a practical one as it may be difficult in some cases due to social, cultural and economic reasons.[39] Obesity is the main risk

factor and has a significant effect in the incidence of breast cancer in postmenopausal women.[41] Dietary modification is an important aspect as a high body fat content is a main component associated with breast cancer risk. A diet, which is high in animal fat, salt and free sugars but low in whole grains, vegetables and fruits increases the risk of cancer,[44] as do to cardiovascular diseases, many other chronic diseases, together with alcohol consumption[45] and cigarette smoking.[59]

For secondary prevention, early detection by breast self-examination and mammography followed by clinical examination reduces the rate of mortality.[31,47] Once the mammographic findings point to suspicion of carcinoma, a fine-needle aspiration and a more detailed histological biopsy are generally carried out to confirm malignancy.[31,47] Factors such as the woman's age, menopausal status, steroid hormone receptors status, tumour features, local spread, lymph node involvement and metastatic status in other parts of the body would determine the options of treatment employed.[5,47,35,60] Decision will have to be made if the cancer can be surgically removed or will require multiple types of treatment such as chemotherapy, hormonal treatment and/or radiation therapy.[47,61]

Once breast cancer is detected, the first-line treatment for the disease is the surgical removal of the tumour often followed by post-operative radiotherapy (exposure to ionising rays such as those emitted by an X-ray tube),[31,35] which helps to prevent the recurrence of the disease. The type of surgical procedure is often related to the size of the tumours with much emphasis on breast conservation and surgical techniques that are more cosmetically acceptable and also psychologically reassuring to the patients.

For small tumours with <2-3 cm diameter, lumpectomy, which is the removal of the lump together with normal tissue from 1 cm surrounding, without removing the overlying skin, and quadrantectomy, which is the removal of the entire quadrant of the breast are the primary choice of treatment.[47] Pre-operative radiation therapy is not preferred for small tumours with no skin involvement and metastasis.[62]

For larger tumours, mastectomy is the general choice of treatment, which is only performed, in exceptional cases of extensive and regionally invasive tumours.[31,5,47] For patients who have had surgery, postoperative treatment (adjuvant therapy) is often recommended to improve the chance of eliminating any remaining cancer cells.[63,61]

1.7 Treatment of advanced breast cancer

In most patients in Western countries, their disease is often being diagnosed in its early stages, 0, I or II. In developing industrialised countries, disease at stages III and IV represent more than 50% of the newly diagnosed cases.[64] Some patients who have a very aggressive form of the disease and do not respond to any form of treatment will die less than 6 months after the diagnosis of the metastatic disease. However, some patients survive for 10 years or even longer with indolent metastatic carcinoma, regardless of the therapy instituted.[31,47]

The two most important treatment modalities for advanced breast cancer are hormonal manipulation and chemotherapy. Before the type of treatment is decided, the patient's hormone dependency status of the tumours has to be diagnosed.[35] However, in advanced HDBC, where metastases of the tumour have already taken place from its primary site to the lymph glands, peri-breast tissues, skin and bones and in more severe cases, the lungs, liver and brain have already taken place and the condition becomes incurable. Therefore further clinical interventions will have no significant impact on the development of the disease. Patients with visceral metastasis have the poorest prognosis, and a much shorter disease free interval.[47] The aim of the treatment for advanced breast cancer is therefore often not a curative measure but a palliative control.

1.7.1 Endocrine Ablation

Ovarian ablation by surgery or irradiation has been an effective treatment in providing tumour regression in around one-third of premenopausal patients with recurrent breast cancer.[47] ER^+ patients show the best response to ovarian ablation. Amongst the non-responders a further 10-20% of patients show remission following adrenalectomy

and/or hypophysectomy.[65] Because of the irreversible nature and unpleasant side effects of such surgical procedures it is preferred to delay ovarian ablation in premenopausal women until the patients show a positive relapse.[35] However, in the management of early breast cancer, a revival call for a prophylactic use of ovarian ablation after mastectomy has been raised in selected premenopausal patients with high risk of relapse.[35,66,60]

In postmenopausal women, the ovaries have ceased to produce estrogens, and hence it would not be effective in carrying out ovarian ablation. Therefore, endocrine ablation therapy becomes the preferred mode of treatment.[67,35] In patients whose tumours are ER^+, adrenalectomy and/or hypophysectomy can be a form of treatment. However, these modes of treatment have subsequently become obsolete because studies have revealed that such ablative treatment did not result in complete elimination of estrogen production,[68] and also due to the innovation of newer and more effective alternative endocrine therapies. (See later)

1.7.2 Chemotherapy

Cytotoxic chemotherapy is another regime widely used in the treatment of patients with advanced breast carcinoma. Chemotherapy could offer substantial palliative benefits, in which large tumours are reduced to operable size tumours so that a breast conservational surgery can be carried out. However, it has the drawbacks of high toxicity and side effects.[35]

The quality of life issues of patients undergoing chemotherapy remain crucially important. Chemotherapy appears to prolong the median survival duration of patients, probably by approximately 1 year. Many single agents such as the anthrapyrazoles, antifolates, gemcitabine, miltefosine, taxanes and vinca-alkaloids are available for more than four decades as cytotoxic treatment.[64] Until recently, doxorubicin was considered to be the most active single agent available for the treatment of metastatic breast cancer, with response rates of 40% to 50% commonly reported. Other active agents widely used include cisplatin, cyclophosphamide, 5-fluorouracil, ifosfamide,

methotrexate, mitomycin- C, mitoxantrone, vinblastine and vincristine.[64]

Initial observation has shown that combination therapy (polychemotherapy) is more effective than single-agent treatment, in terms of response rate, response duration and survival.[69] Polychemotherapy as adjuvant treatment for early breast cancer has produced significant reductions in recurrence and mortality, with a statistically significant trend towards greater benefits in patients aged less than 50 years.[70] FAC (5-Fluorouracil/doxorubicin/cyclophosphamide), FEC (5-Fluorouracil/epirubicin/ cyclophosphamide), AC (doxorubicin/cyclophosphamide), NFL (5-Fluorouracil/ mitoxantrone/leucovorin), MMM (mitozantrone/methotrexate/mitomycin C) and EP (etoposide/cisplatin) are some of the combination drugs used for this purpose, which produce a response rate of 40-80% in patients with no prior cytotoxic treatment.[64,69] These combination drugs are given every 3-4 weeks in several courses over a period of 6 months. In resistant patients combination therapy may be followed by a course of vinblastine/methotrexate, paclitaxel or vinorelbine and then by a further course of 5-Fluorouracil/folinic acid or vinblastine/mitomycin. The major disadvantage of polychemotherapy is that treated patients eventually develop tolerance due to an induction of a P_{170} membrane glycoprotein and the disease progresses even further.[64]

With the newer cytotoxic agents introduced lately show that anthracycline-containing regimens were found to be more effective than combinations without anthracyclines, but overall, it is not clear whether combinations are superior to high-dose single-agent anthracyclines.[71] The addition of anthracyclin has extended disease-free interval and overall survival rates in both premenopausal and postmenopausal women, including those with ER^+ tumours.[70] Also, despite the development of the cyclophosphamide/doxorubicin/5-fluorouracil regimen, no major improvements on combination regimens have been achieved until the discovery of the taxoids. These new agents have substantial activity against metastatic breast cancer. The results of phase II studies have suggested that of these agents used at the recommended doses, docetaxel (Taxotere, Rhone-Poulenc Rorer) may be the most active. Docetaxel has been given at a standard dose of 100 mg/m^2 over 1 hour. Recent results from phase III

studies in which individual studies with docetaxel and paclitaxel (Taxol; Bristol-Myers Squibb) have been compared with standard therapies, have indicated that docetaxel is the most active single agent against metastatic breast cancer.[64,69]

1.7.3 Endocrine Therapy

It is now over 100 years since it was first recognised that altering the endocrine environment can be valuable in patients with inoperable breast cancer. Endocrine therapy represents one of the most effective palliative and adjuvant treatments for breast cancer, in particular in postmenopausal patients.[70] It aims to abolish or reduce the stimulatory effects of estrogens on hormone dependent tumours. The choice of endocrine agent for breast cancer depends on the menopausal status of the patient, the stage of disease, prognostic factors, and the toxicity profile of the agent. Endocrine therapies are typically given sequentially, with the least toxic therapy given first.[70]

The elucidation of the biosynthetic routes and mechanism of action of steroid hormones has helped to define the role of hormones on tumour growth. The discovery of ERs within the breast tumours has led to a greater understanding of the disease and conferred selectivity on the administration of endocrine therapy. Also, new agents have been discovered, which offer effective and safer alternatives to the conventional methods of treatment. Therefore, we are specifically interested in synthesising new drugs with a novel pharmacological action, which are effective and well tolerated by patients for use in the endocrine therapy.

In 1896, Beaston had laid the foundation for investigation into the role of endocrine treatment in breast cancer[72] by demonstrating that oophorectomy/ovariectomy (surgical removal of ovaries) could result in a significant regression of tumour in premenopausal women with advanced breast cancer. Despite such demonstration ovariectomy had not been widely practised for some time since only about one-third of the patients were found to respond to the surgery and the response was of a short duration, up to a year.

However, a renewed interest in endocrine therapy flourished in the early 1940's, when

Huggins showed the benefit of orchidectomy (surgical removal of testis) in men with metastatic prostate carcinoma.[73] As a consequence, oophorectomy conducted either by surgery or radiation had become a standard treatment for breast cancer in women. Unexpectedly, recurrence of the disease was still common in these operated patients. The failure to cure the disease was subsequently attributed to the adrenals, where the precursor of estrogens is produced after surgery. It is now known that the androgens synthesised by the adrenals are converted into estrogens in other tissues of the body. Since synthetic glucocorticoids were not available at that time, the removal of the adrenals was not practised, since such surgery would have led to Addisonian crisis due to insufficient adrenal hormones.

When synthetic glucocorticoids finally became available in 1952, adrenalectomy was shown to produce good remission in postmenopausal women. Among those non-responders to oophorectomy, around 10-20% will show remission following adrenalectomy and/or hypophysectomy (surgical removal of the pituitary glands).[74,68] Experiments, which were carried out in animals, have shown that the growth of some mammary tumours may be dependent on pituitary hormones, growth hormones and/or prolactin. However, the clinical results achieved with hypophysectomy were similar to those with adrenalectomy.[68]

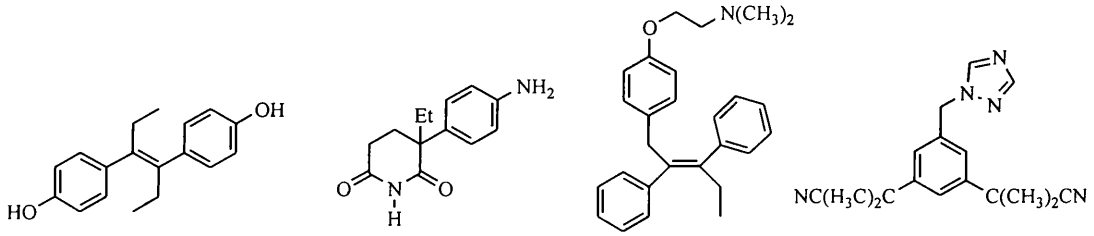
As an alternative to these ablative procedures, the administration of pharmacological doses of androgens such as testosterone were used in the 1940's to antagonise the action or the biosynthesis of estrogens. Even though this kind of therapy showed tumour regression, long term use of these hormones had led to virilising side effects. Administration of antiestrogens[75] and high doses of estrogens[76] had also been tried and tested.

1.8 Drugs used for the treatment of breast cancer

Once Haddow and co-workers had shown paradoxically that large doses of estrogens could cause tumour regression in 1944, several synthetic estrogens then became available.[76] The most commonly used synthetic estrogen then was diethylstilbestrol

(DES) **(1-9)** (Figure 1.6). Although the mechanism of action is unclear, the most likely explanation for the clinical effects is that a large dose of DES causes blockage of estrogen response, rather than stimulation as caused by smaller doses of naturally occurring estrogens. The remission rates were around 35%, but prolonged use had led to uterine bleeding, cardiovascular toxicity, nausea and vomiting.

Nowadays, the most commonly used hormonal agents for treating advanced breast cancers are aminoglutethimide (AG) (Orimeten) **(1-13)**, tamoxifen (Nolvadex) **(1-14)** and 3rd generation AR inhibitor anastrazole (Arimidex) **(1-15)** (Figure 1.6). Tamoxifen is an anti-estrogen and the other two inhibit the aromatase enzyme complex, which is responsible for the conversion of androgens into estrogens.[70] AG inhibits several enzymes involved in the adrenal steroid synthesis and has been found to act as a "medical adrenalectomy" since its clinical results observed are similar to those achieved with surgical methods.[77]



Diethylstilbestrol **1-9** | **Aminoglutethimide (AG)** **1-13** | **Tamoxifen** **1-14** | **Anastrazole** **1-15**

Figure 1.6 : Drugs used for the treatment of breast cancer

The long-term clinical effects of AG and tamoxifen are of limited nature. The mean duration of response is about 18 months for AG and about 20 months for tamoxifen. AG lacks selectivity for the AR enzyme and it is toxic.[78] AG also inhibits other cytochrome P_{450} enzymes in particular cholesterol side chain cleavage enzyme. For this reason a corticosteroid is often administered concurrently with AG to counteract the resulting adrenal hypo function. The side effects of tamoxifen are relatively mild and uncommon. Nausea, vomiting, lethargy, drowsiness, oedema, weight gain, vaginal

bleeding, rash and hot flushes are occasionally encountered. Because of its low toxicity, tamoxifen is used as the first-line treatment for women with advanced breast cancer. AG is being regarded as the second line therapy because a significant proportion of postmenopausal patients who developed tolerance to tamoxifen will respond to AG only if they have not been treated with AG beforehand.

The new drug anastrazole **(1-15)** is a comparatively simple, achiral derivative of benzyltriazole (2,2'-[5-(1H-1,2,4-triazol-1-yl-methyl)-1,3-phenylene]bis(2-methyl propiononitrile), that inhibits human placental aromatase enzyme with an IC_{50} value of 15 nM.[79] It is potent and selective with maximum activity elicited at about 0.1 mg/Kg. There are no other pharmacological effects or interference with the steroid hormones production by the adrenal glands being observed.[80] It is extensively metabolised after oral administration and the metabolites are excreted predominantly in urine.[81] It has a half-life of 30-60 hrs and is considered to be the best inhibitor available to date for the treatment of advanced breast cancer. [82]

1.9 Antiestrogens or Selective Estrogen Receptor Modulators (SERMS)

Antiestrogens were first used as fertility drugs. This was based on the fact that E2 inhibits the secretion of gonodotrophic hormones LH and FSH by a feedback mechanism, which results in the production of one single ovum in every menstrual cycle and hence prevents overlapping pregnancies. Antiestrogens block this inhibition of E2 in infertile women by occupying ER in the hypothalamus resulting in an excessive LH and FSH production and hence facilitating multiple pregnancies.[70]

For the past 25 years antiestrogens have been used as the first line treatment for HDBC. Rather than relying on the removal of endocrine glands, which will ablate many important hormones in addition to estrogens, antiestrogens interfere with estrogen's interaction with its target cells by antagonising E2 at its receptors. The triphenylethylenes and the aminoether derivatives of stilbene are the two classes of non-steroidal antiestrogens commonly used as anti-tumour agents in ER^+ estrogen-dependent mammary carcinoma. These non-steroidal drugs have been shown to

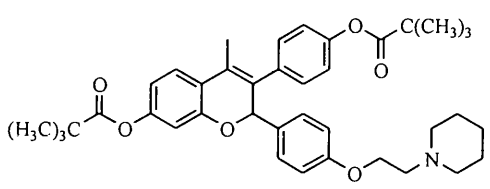
partially antagonise E1 and E2 induced uterotrophic activity in mice.[83]

Tamoxifen is the most widely prescribed endocrine therapy for breast cancer, which has both antiestrogenic and estrogenic activity. The antiestrogenic activity accounts for its efficacy against breast cancer, while the estrogenic activity is considered to be associated with positive effects on bone mineral density and lipid profiles and a proliferative effect on the endometrium in some women. Tamoxifen inhibits the synthesis of estrogen-regulated proteins by blocking the G_1 phase of the cell cycle. This cis- isomer of tamoxifen is estrogenic rather than being an estrogen antagonist.

Tamoxifen has a good tolerability profile. It benefits breast cancer patients by prolonging the overall disease-free survival and reducing the incidence of contralateral breast cancer. These known benefits of tamoxifen far outweigh the risk of endometrial cancer in tamoxifen-treated patients with breast cancer.[84] Tamoxifen is currently being tested as a chemopreventive agent in patients with high risk of developing breast cancer. However, a number of studies have shown genotoxic and carcinogenic effects, which are thought to be related to oxygen radical overproduction during tamoxifen metabolic activation.

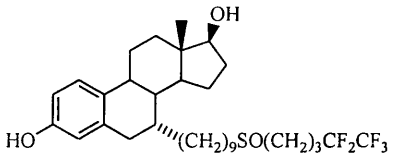
In early breast cancer, tamoxifen can produce significant benefits, both statistically and clinically, in terms of reduction in relative risk of relapse or death in all patient subgroups (i.e. ER status, aged < or > 50 years) except in premenopausal women with ER^- negative tumours. The major benefit, however, is seen in women over 50 years old with ER^+ tumours. The results of randomized trials have suggested that the optimum duration of tamoxifen therapy is at least 5 years. Recent data has also suggested a possible synergism between tamoxifen and chemotherapy in the treatment of early breast cancer in post-menopausal women. Although, tamoxifen is the first choice endocrine therapy for all stages of breast cancer, the drug cannot be considered to be a cure, as drug resistance will eventually develop by the loss of ER.

A number of pure antiestrogens (nonestrogenic) have been discovered that can either be administered orally [e.g., EM-800 **(1-16)**] or by injection [e.g., ICI 182,780 – Faslodex **(1-17)**][85] (Figure 1.7). In preliminary clinical studies, Faslodex has been shown to be an effective second-line therapy after tamoxifen failure.[85]



EM 800

1-16



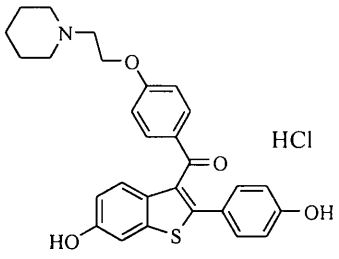
ICI 182 780 (Faslodex)

1-17

Figure 1.7 : Structures of some antiestrogens

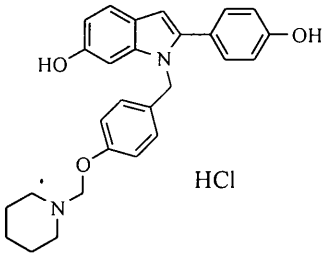
It has now been discovered that antiestrogens also act as selective estrogen receptor modulators (SERMs). They are competitive inhibitors of estrogen binding at estrogen receptors α and β, and have become important weapons in the prevention and treatment of HDBC.[86] SERMs are also shown to represent a possible alternative to HRT,[87] which is the first and probably the sole therapeutic approach for the prevention and treatment of medical postmenopausal disease.

A benzothiophene, raloxifene hydrochloride (LY-139481, Evista®) **(1-18)** (Figure 1.8) binds to the estrogen receptor and shows tissue-selective effects such as estrogen-agonist effects on bone and lipids and estrogen antagonist effects on the breast and uterus. In addition to its well-established effects on osteoporosis, it can also be used as a replacement to HRT. Recent preclinical and clinical findings suggest that raloxifene also possesses cardioprotective properties without an increased risk of cancer or other side effects.[88] Newer drugs, ERA-923 **(1-19)**,[89] the coumestrol derivative **(1-20)**,[90] the isoflave-3-ene derivative **(1-21)**[91] have also shown to possess SERM properties (Figure 1.8).



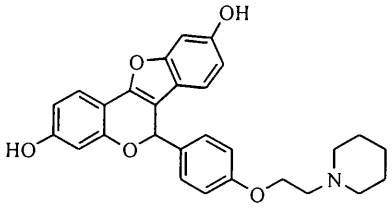
Raloxifene Hydrochloride

1-18



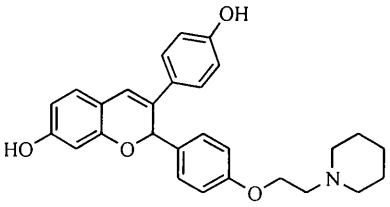
ERA 923

1-19



Coumestrol Derivative

1-20



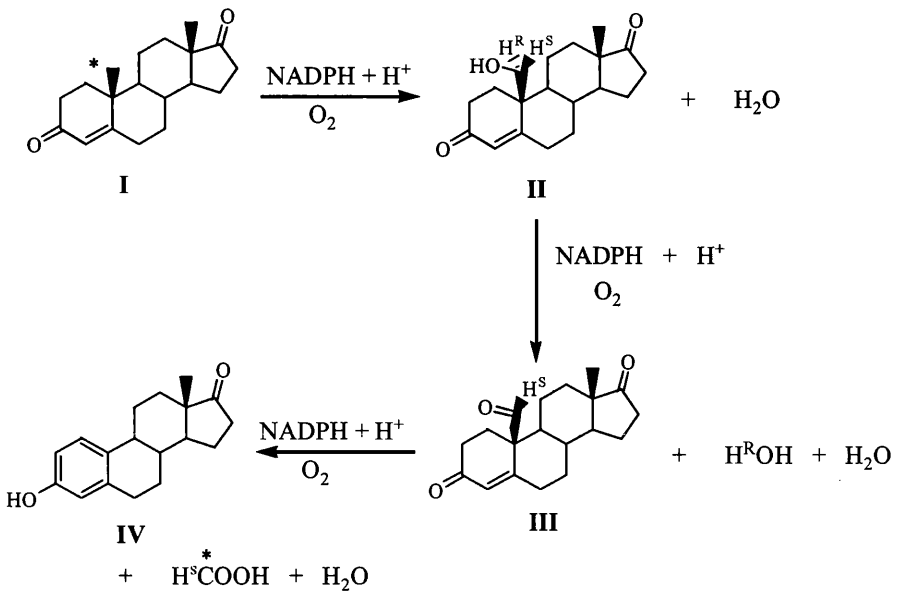
Isoflav-3-ene Derivative

1-21

Figure 1.8 : Structures of some SERMS

1.10 Aromatase enzyme

The three key enzymes that are involved in the synthesis of estrogens in breast tumours are important targets for therapeutic intervention, such as aromatase (AR), estrone sulphatase (E1-STS) and 17β-hydroxysteroid dehydrogenase (17β-HSD). AR is a membrane-bound enzyme complex consisting of a cytochrome P_{450} haemoprotein (a 55-kDa protein of 503 amino acids) and a ubiquitous electron-donating flavoprotein, NADPH-cytochrome P_{450} reductase.[92] The C-19 androgen substrates androstenedione and testosterone bind to the P_{450} and AR catalyses the aromatisation of the androgen A ring and loss of the C19 methyl group to yield E1 and E2[93] extragladularly in peripheral tissues such as adipose, muscle, liver and skin[94] (Figure 1.9). AR has also been described in other parts of the body such as brain (hypothalamus, amygdala, hippocampus),[95] ovary, testis, adrenal, breast, placenta and bone marrow.[96]



I II III IV

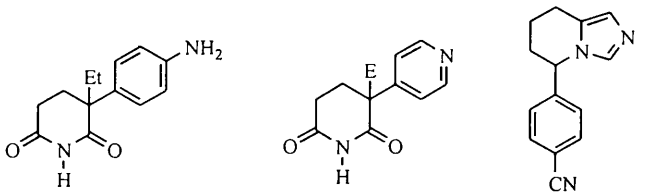
Figure 1.9 : Aromatisation of androgens to estrogens **(I)** Androstenedione or testosterone substrates **(II)** and **(III)** intermediates of the reaction **(IV)** products of E1 and E2.

AR represents the rate-limiting step in the production of estrogen and expressed at a higher level in human breast cancer tissue than in normal breast tissue. This has been determined using enzyme activity measurement and immunocytochemistry. Cell culture, animal experiments using aromatase-transfected breast cancer cells, and transgenic mouse studies have demonstrated that *in situ* produced estrogen plays a more important role than circulating estrogens in breast tumour promotion. In addition, tumour AR has been shown to stimulate breast cancer growth in both an autocrine and a paracrine manner. Gene transcriptional studies have revealed that AR promoter switches from a glucocorticoid-stimulated promoter, I.4, in normal tissue to cAMP-stimulated promoters, I.3 and II, in cancerous tissue. Suppression of *in situ* estrogen biosynthesis can be achieved by the prevention of AR expression or by the inhibition of AR activity in breast tumors.[97] While the control mechanism of AR expression in breast cancer tissue is not yet fully understood, AR-inhibitor therapy is considered for second-line treatment in patients who fail antiestrogen therapy. 20-30% of the patients who fail antiestrogen treatment respond to AR-inhibitor treatment.

1.11 Aromatase inhibitors

The benefits of more recent therapies, such as the new generation of AR inhibitors, appear to relate to better tolerability and more convenient administration. It is now recommended that the majority of patients with advanced breast cancer should receive endocrine therapy and treated with an AR inhibitor as their first mode of treatment, although it is postmenopausal patients who are likely to gain most benefit from this approach.

AG **(1-13)** (Figure 1.6) is the first AR inhibitor used clinically for HDBC. Because of the similarity of the P450 enzymes, selectivity is important; nonselective AR inhibitors can affect enzymes controlling the production of other steroids and lead to significant side effects. Since AG lacks selectivity to AR enzyme and inhibits several other P450 enzymes, it is co administered together with a glucocorticoid.[98] AG also possesses other side effects such as depression and rash, which limit its usefulness and hence came the second generation AR inhibitors such as rogletimide **(1-22)** and the most potent competitive inhibitor fadrozole[99] **(1-23)** (Figure 1.10).



Aminoglutethimide (AG) **1-13**

Rogletimide **1-22**

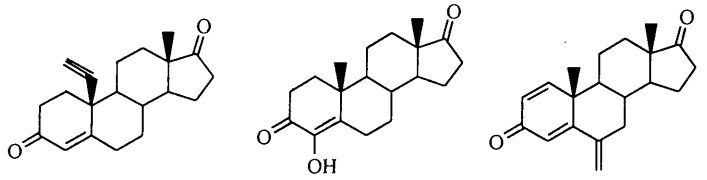
Fadrozole **1-23**

Figure 1.10 : 2nd Generation aromatase inhibitors

AR inhibitors represent a heterogeneous family of compounds able to provide more or less selective inhibition of AR enzyme. Depending upon the way in which the inhibitor binds to the enzyme active site, AR inhibitors can be classified as Type I or suicide inhibitors, and type II inhibitors. The steroidal analogues compete rapidly with the substrate for the active site of the enzyme by initiating a time-dependent reactive process resulting in either covalent or very tight binding of the inhibitor to the enzyme

and causing its inactivation. Because the reaction occurs at the enzyme active site, the inhibitors are quite specific and the compound also has lasting effects, since new enzyme synthesis is required to overcome this type of inhibition. Thus, the continued presence of the drug to maintain inhibition is not necessary and the chance of toxic side effects to the patient will therefore be reduced.

The steroidal derivatives plomestane **(1-24)**, formestane (4-hydroxyandrostenedione) **(1-25)** and exemestane (6-methyleneandrosta-1,4-diene-3,17-dione) **(1-26)** are type I inhibitors, which bind to the steroid recognition site of the enzyme and hence activate the catalytic mechanism (Figure 1.11). The intermediate formed then covalently binds to the neighbouring amino acids leading to an irreversible inhibition of the enzyme.[100,101]



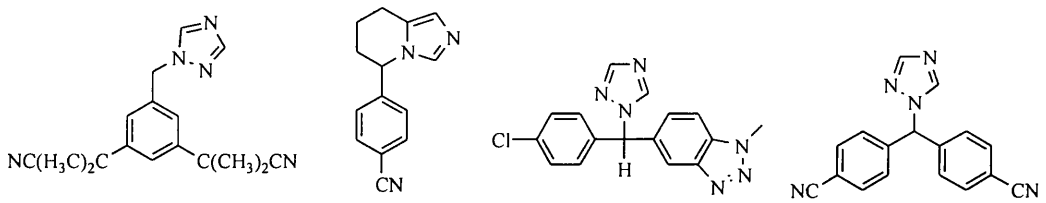
Plomestane **1-24** **Formestane** **1-25** **Exemestane** **1-26**

Figure 1.11 : Type I aromatase inhibitors

Formestane is one of the first drugs on this type to be used clinically and has been demonstrated to be as effective as tamoxifen in first-line therapy, but reduces patient complience[102] due to poor oral bioavailability.[103] Due to its high first-pass metabolism, this drug is administered intramuscularly rather than orally. Formestane and exemestane have shown to reduce the circulating levels of E1 and E2 significantly, and have been shown to be active in 20% of patients pretreated with tamoxifen. Moreover, exemestane was also effective in patients pretreated with type II inhibitors, of which the parent drug is AG. Even though exemestane shown to be an effective AR inhibitor, the most commonly occurring adverse events such as nausea, hot flushes, fatigue, increased sweating and dizziness were observed.[104] Formestane, the first selective AR

inhibitor, has been shown to reduce serum estrogen concentrations and cause complete and partial responses in approximately 25% of patients with hormone responsive disease who have relapsed from previous endocrine treatment.[105]

The imidazole and triazole derivatives, anastrazole (Arimidex-Zeneca) **(1-15)**, vorozole **(1-27)**, letrozole[106] (Femara-Novartis) **(1-28)**, fadrozole **(1-23)** and miconazole are the third generation type II drugs, which are non-steroidal and compete reversibly with androestenedione for the enzyme active site (Figure 1.12). In these inhibitors, the basic nitrogen atom ligates with the iron atom at the centre of the heam moiety of the enzyme while the rest of the structure of the inhibitor aligns more or less closely with the steroid binding domain.[107]



Anastrazole **1-15** | **Fadrozole** **1-23** | **Vorozole** **1-27** | **Letrozole** **1-28**

Figure 1.12 : Type II aromatase inhibitors

Anastrazole and letrozole are the first selective, oral and non-steroidal AR inhibitors, which are licensed for the treatment of advanced breast cancer in postmenopausal women, where tamoxifen or other antiestrogen therapy has failed. These inhibitors have recently been found to suppress plasma and urinary estrogens by more than 95% in breast cancer patients.[101] Anastrazole and letrozole have both shown tolerability and efficacy advantages over standard treatments in postmenopausal women with advanced breast cancer. There are convincing reasons why the new generation of AR inhibitors has advantages over tamoxifen. For instance, from their agonist properties, the effects on the endometrium and tumour stimulation seen with tamoxifen would not be expected, nor would the visual disturbances that have been associated with the triphenylethylene compounds, including tamoxifen. Of those AR inhibitors,

anastrazole is now used widely in the UK, since it has been shown to be more effective, less toxic and better tolerated than the standard therapies used clinically.[108]

1.12 Current Therapies in Development

The past decade has witnessed an accelerated phase in the development of new drugs and strategies that have documented activity and/or a major interest in breast cancer. Apart from the conventional methods of treatments employed, the newly emerged therapies have now broadly revolutionised. Researchers are concentrating more on the interception of specific pathways of carcinogenesis and studying the various strategies associated with malignant-cell behaviors such as uncontrolled proliferation, unregulated cell division, cell survival/immortality, sustained angiogenesis, dissemination and metastasis.

1.12.1 Cyclin-Dependent Kinases and inhibitors

Recently, a cellular process has captured attention for therapeutic intervention. It is the inhibition of a key class of cell cycle proteins, cyclin-dependent kinases (CDKs) that control and drive the cell cycle and are present in the growing cell at constant concentration but much of the time they are inactive. CDKs are enzymes that phosphorylate the serine and threonine amino acids using adinosine trisphosphate (ATP) and activate host proteins.[109] For these kinases to be active they must first attached to a protein, cyclin.[110] The progressions through the individual phases of the cell cycle are indeed driven by CDKs.[111] There are at least 9 CDKs and more than twelve different cyclin families have been identified so far.[109] Normal cells undergo cell division at a controlled and limited rate. Malignant cells escape from the normal control mechanisms that regulate the rate and frequency at which they divide. It has been found that tumour cells appear to have unregulated CDKs, which trigger cell cycle progression and cell division. In addition, tumours have frequently lost the control over the prevention of replication of defective cells.

The key CDK inhibitors are: (1) Tyrosine kinase inhibitors, such as the 3-substituted indolinone SU 5416[112] and the substituted anilinoquinazoline ZD 1839.[109] (2) Fusudil,

a diazepine developed as a vasodilator, which inhibits smooth muscle cell proliferation and DNA synthesis.[109] (3) Staurosporine, a protein kinase C inhibitor.[113] (4) Flavopiridol, a most advanced flavone currently in Phase II clinical trials.[114] (5) Olomoucine, a dose-dependent inhibitor of the G1/S phase and G2/M/G1 transitions in cell cycle.[115] (6) Roscovitine, a second generation 2- and 9-substituted purine, which causes a complete cell cycle block[116] and (7) Purvalanol B, which shows selectivity to CDK4/cyclin D1 in addition to other kinases.[109]

1.12.2 Oncogene targets and inhibitors

Cancer arises from the aberrant growth of cells that have sustained mutations in genes controlling cell proliferation and survival. The great majority of human breast cancers arises from epithelial cells and genetic analysis of tumour cells obtained from patients have revealed several commonly mutated genes. Determining the expression of those genes and targeting and manipulating them is a new approach to the treatment.

Mutations in the *p53* tumour suppresser gene occur in majority of the tumours.[117,118] Breast cancer also frequently carries mutations that deregulate the retinoblastoma protein (*pRB*) pathway including loss of expression of *pRB*[119] or $p16^{INK4a}$[120] or amplification or overexpression of cyclin D1[121,122,123] or cyclin E.[124] Breast carcinoma cells also commonly acquire alterations in the *Ras*-signaling pathway,[125] which may occur by several mechanisms, most notably amplification and overexpression of the *HER-2/neu* gene.[126] *C-myc*[118,127,128] and *c-erbB-2*[118] genes are also frequently seen amplified or overexpressed. The deregulation of *Wnt* expression patterns and signaling,[128] and *bcl-2* expression[129] also play a role in breast cancer

These individual genetic mutations have been catalogued in numerous breast carcinomas, none is involved universally in all human breast cancers, and the number of mutant genes that coexist in the genome of a naturally arising breast tumour cell is unknown. As a consequence, it has been impossible to know how many mutant genes are required to convert a normal human mammary epithelial cell (HMEC) into a

tumour cell. Therefore, extensive studies are being carried out at present to identify the specific combinations of mutations required to form breast carcinoma cells.[130]

It has been identified that 25% of patients with *HER-2/neu*-positive metastatic breast tumors respond favorably to trastuzamab (Herceptin) treatment.[131] Weekly therapy with a combination of trastuzumab and paclitaxel in women with *HER-2*-normal and *HER-2*-overexpressing metastatic breast cancer showed significant activity. Although the therapy was relatively well tolerated, attention must be given to assess the cardiac function.[132]

1.12.3 Targeting telomerase enzyme and inhibitors

Telomeres are specialised DNA-protein structures consisting of a large number of tandem repeats of TTAGGG at the end of eukaryotic chromosomes; these are lost with each cell division and the telomere becomes short in length.[133] In this way, the length of the telomere indicates its age to the cell. The cell is programmed so that, after a certain number of divisions and shortening of telomeres causing chromosomal instability, it will enter apoptosis.[134] Telomerase is a ribonucleoprotein complex with reverse transcriptase activity, which adds telomeric repeats to the 3' end of telomeric DNA. Tumour cells contain an abundance of telomerase, which is specifically activated in most malignant tumours but is usually inactive in normal somatic cells.[135] Telomerase enzyme causes the aging telomeres to re-grow. This telomere stabilisation by telomerase can lead to unlimited cell proliferation.[136] Thus, the cell escapes the controls that regulate ageing and prevent defective cells from multiplying and dividing indefinitely.

Human telomerase reverse transcriptase (hTERT), which has been purified as human telomerase catalytic subunits, regulates telomerase activity.[137] Recent observations support the notion that telomerase expression is essential for the formation of human tumor cells. The expression pattern of hTERT is a rate-limiting determinant of the enzymatic activity of human telomerase.[138]

Retinoic acid and its analogues, such as all-trans retinoic acid, 9-cis retinoic acid and 13-cis retinoic acid, were shown to be effective inhibitors of telomerase activity in human breast cancer MCF-7 cells in a wide range of concentrations investigated.[139(a)] The inhibition was preceded by a reduction of hTERT mRNA expression indicating that the anti-breast cancer activity of retinoic acid could be mediated by its ability to down-regulate the expression of hTERT telomerase gene.[139,140] Also, a time-dependent and consistent decrease of telomerase activity in the tumours was observed with 4-(hydroxyphenyl)retinamide, a known inhibitor of mammary carcinogenesis.[93] A DNA-methylating prodrug, temozolomide, has also exhibited antitumour activity and has been selected for clinical trials.[141] Therefore, in addition to the percentage of proliferating cells, telomerase activity could also be used as an endpoint in breast cancer chemotherapy.

1.12.4 Tumour vasculature as a target and inhibitors

Vascular endothelial growth factor (VEGF), a potent angiogenic factor, has been reported to be associated with a poor prognosis in primary breast cancer and in several other cancer types. Angiogenesis inhibition is an extremely promising novel approach in cancer prevention, because tumours are dependent on the formation of new blood vessels to support growth and metastasis. Many tumours produce large amounts of VEGF, which signals normal blood vessels to grow. Binding of VEGF to its receptors activates signal–transduction pathways, which results in the formation of new blood vessels that supply the tumour with nutrients and oxygen.

VEGF-C is a specific ligand that induces lymphangiogenesis and determines the progression of breast cancer. Immunohistochemical analysis revealed that VEGF-C was overexpressed in 40% of breast cancer specimens, but not in adjacent normal mammary glands.[142] VEGF levels were not related to age or menopausal status but were negatively related to the cytosolic levels of ER and PR.[143] Patients with high or intermediate levels of VEGF usually show a poor rate of response, compared with patients with low tumor-VEGF levels. Higher levels of VEGF were also associated with a short progression-free survival and post-relapse overall survival.[143] Therefore,

tumor VEGF level in a patients is an important independent marker that helps selecting individual patients who may benefit from treatments with antiangiogenic agents combined with conventionally used drugs.

Recent studies have revealed that silymarin, a naturally occurring flavonoid antioxidant, exhibits anti-cancer effects against several epithelial cancers. Silymarin resulted in a dose-dependent decrease in the secreted VEGF level in conditioned media without any visible change in cell morphology.[144] Vitamin E succinate, the most potent antitumour analogue of vitamin E, was found to inhibit VEGF gene expression in MDA-MB-231 cells and involves in the inhibition of tumor angiogenesis hence inhibits the growth of breast cancer cells *in vitro* and *in vivo*.[145] Thalidomide is another agent inhibited the processing of mRNA encoding peptide molecules including tumour necrosis factor-alpha (TNF-alpha) and the angiogenic factor VEGF.[146] It has also been reported that tamoxifen could also be used since VEGF expression was significantly correlated with estrogen receptor status and inversely correlated with tumour grade.[147] These agents potentially possess antiangiogenic ability that may critically contribute to its cancer chemopreventive efficacy.

1.12.5 Cyclooxygenase 2 as a target and inhibitors

Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins and into several metabolites, some of which have been identified as modulators of mitogenesis and apoptosis.[148] Elevated prostaglandin production is a common feature of human malignancies. This activity has often been attributed to increased metabolic activity of the COX-1 and COX-2 enzymes. COX-2 is highly expressed in a number of human cancers and cancer cell lines. COX-2 is only expressed in tumours resulting from the transplantation of metastatic cell lines whereas COX-1 is detected in both metastatic and nonmetastatic tumors.[149]

Although COX-2, the inducible isoform, is regularly expressed at low levels in colonic mucosa, its activity increases dramatically following mutation of the APC (adenomatous polyposis coli) gene suggesting that beta-catenin/T-cell factor mediated

Wnt-signaling activity may regulate COX-2 gene expression. In addition, hypoxic conditions and sodium butyrate exposure may also contribute to COX-2 gene transcription in human cancers.

The development of selective COX-2 inhibitors has made it possible to further evaluate the role of COX-2 activity in colorectal carcinogenesis. The recent marketing of two selective COX-2 inhibitors, celecoxib and rofecoxib is remarkable considering that COX-2 was only discovered eight years ago as a growth factor- and cytokine-inducible gene.[150] Studies indicate that the use of non-steroidal anti-inflammatory drugs (NSAIDs) may enhance the antitumour activity of cancer chemotherapeutic agents and reduce the risk of many cancers. The best-known function of NSAIDs is to block the COX-1 and COX-2 enzymes by altering the metabolism of arachidonic acid and, subsequently, prostaglandins.[151] Recent studies have revealed that nearly complete inhibition of prostaglandin synthesis and cell growth occurs in the presence of either indomethacin, which inhibits both COX-1 and COX-2 isoforms, or NS 398, which is selective for the COX-2 isoform.[149] It is now anticipated that pre-treatment with selective COX-2 inhibitors may be useful in the prevention of multi-drug resistance in response to cancer chemotherapy.

1.12.6 Immunotherapy of breast cancer

Triggering the immune system to attack malignant diseases, such as breast cancer, involves three main approaches. The first is the passive immunotherapy, which involves the administration of antibodies such as Herceptin or T-lymphocytes with defined specificity. A second approach is based on the transfusion of donor lymphocytes to patients in whom a chimerism has been obtained following a previous allogenic bone marrow transplantation; injection of T-lymphocytes may be capable of killing residual tumour cells. A third strategy attempts to induce an immune response against cancer cells *in vivo* using various immunogens, such as *HER-2* or *MUC-1* peptides, dendritic cells loaded with tumour peptides, tumour lysates or RNA and autologous or allogenic tumour cells genetically modified to increase their immunogenicity.[152]

The other recent therapeutic approaches behind uncontrolled proliferation, unregulated cell division, apoptosis and sustained angiogenesis will be discussed in Chapter 7 in detail.

CHAPTER 2

CHAPTER 2

2.0 Sulphatase Enzymes

Sulphatases are a group of hydrolytic enzymes that catalyse the conversion of various sulphated compounds to their various unconjugated derivatives (Figure 2.1). To date, eleven different mammalian sulphatases, such as galactose-3-sulphatase (Arylsulphatase A or ASA), *N*-acetylgalactosamine-4-sulphatase (Arylsulphatase B or ASB or 4-S), steroid sulphatase (STS), arylsulphatase C, arylsulphatase D, arylsulphatase E, arylsulphatase F, *N*-acetylgalactosamine-6-sulphatase (GAL6S), *N*-acetylglucosamine-6-sulphatase (GLU6S), iduronate sulphatase and sulphamidase have been identified, eight of which are in lysosomes and three are associated with microsomal membranes.[153] Nine members of this family of enzymes have been isolated in the humans and the corresponding encoding genes have also been identified.

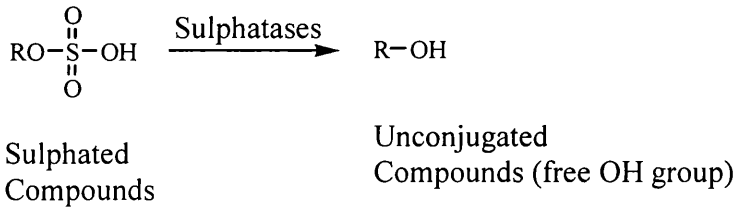


Figure 2.1 : General reaction catalysed by sulphatase family

The natural substrates of human sulphatases have varying degrees of structural complexity, from simple aromatic molecules such as *p*-nitrocatechol and 4-methylumbelliferyl sulphate to more complex molecules such as 3-hydroxysteroid sulphates and glycosaminoglycan sulphates. Sulphated glycosaminoglycans, glycolipids, glycoproteins and hydroxysteroids are hydrolysed by a group of sulphatases, each of which has exquisite specificity towards its individual substrate *in vivo*. E1-STS is a microsomal enzyme, which removes the sulphate group from sulphate esters of phenol or 3β-hydroxysteroids.[154] All these sulphatases whose sequences have been determined so far show that the amino acid residues at their respective active sites are conserved.[155]

STS and aryl sulphatases A, B and C are from distinct subgroup of human sulphatases that possess similar hydrolytic activity toward aryl sulphates. Recently, many studies have focused on the study of STS, the enzyme that regulates the levels of active estrogens and androgens in human target organs and steroidogenic tissues. STS has been suggested to play a key role in endocrine-related diseases such as breast, endometrium and prostate cancers. Aryl sulphatase A and B are lysosomal enzymes, hydrolysing cerebrozide sulphates and dermatan sulphates, respectively. Aryl sulphatase C and STS are located in microsomes and act on 3-hydroxysteroid sulphates.

Different inherited diseases are known to be caused by a deficiency of distinct sulphatases leading to a tissue specific accumulation of the non-degradable sulphuric acid ester. Deficiency of ASA leads to metachromatic leukodystrophy (MLD). A reduced level of ASB leads to Hunter syndrome (mucopolysaccharidosis II), a rare X-linked disorder of mucopolysaccharide metabolism that typically progresses to severe mental retardation.[156] Mucopolysaccharidosis (MSP) type VI is a lysosomal storage disease with autosomal recessive inheritance involving degradation of dermatan sulphate and chondroitin 4-sulphate.[157] Morquio disease (MPS IVA) is an autosomal recessive disorder caused by a deficiency of *N*-acetylgalactosamine-6-sulphatase activity, where patients commonly present in early infancy with growth failure, but milder forms have also been described.[158] Deficiency of all known sulphatases leads to a rare disorder multiple sulphatase deficiency (MSD), which is caused by the failure of an essential post-translational modification of a specific active site cysteine residue to oxo-alanine (formylglycine-FGly)[159,160] (Scheme 2.2).

Previous studies have indicated that aryl sulphatase C and STS are distinct enzymes encoded by different genes and have different electrophoretic mobilities.[161] However, transfection of the cDNA encoding for the placental STS in to COS-1 cells resulted in a protein that hydrolyses both phenolic and 3β-sulphated steroids suggesting that they are both the same enzymes, a transmembrane protein located in the endoplasmic

reticulum (Er) and consisting of 587 amino acids.[154,162]

STS play a major role in steroid metabolism. They are involved in the regulation of many physiological processes such as hypertension,[163] immune response, some reproductive functions, the formation of neurosteroids in the CNS and memory enhancement.[164] STS deficiency was found to associate with allergic diseases (bronchial asthma, allergic rhinitis, or atopic dermatitis).[165] STS is also suspected to be the cause of the complex syndrome associating hypogonadotrophic hypogonadism, X-linked ichthyosis and renal malformation. The absence of the gene encoding STS accounts for the genetic disorder, X-linked ichthyosis, whereas the absence of the Kallmann syndrome gene accounts for hypogonadism.[166] X-linked ichthyosis causes the accumulation of STS in various organs and cells and causes early puberty. Serum STS levels start to increase in early infancy, peak at puberty, remain elevated in adults and decrease slightly in the elderly.[167]

2.1 The role of steroid sulphatase in estrogen synthesis in breast cancer

In premenopausal women, the ovaries provide the major source of estrogens (E2 and E1). These hormones are synthesised in the granulosa cells of the ovarian follicles from androgens (testosterone and androstenedione for E2 and E1, respectively). In postmenopausal women, with the cessation of ovarian activity, estrogens continue to be produced from the adrenal glands *via* the aromatisation of androstenedione to E1 by the AR enzyme complex, which is present in the peripheral tissues such as muscle, adipose and in normal and malignant breast tissues.[14,13,15] It is therefore anticipated that patients with HDBC should benefit from estrogen ablation elicited by an effective inhibition of the AR enzyme. As a result, a large number of AR inhibitors have been produced and some of them are now in clinical use. However, anastrazole **(1-15)** (Figure1.6, Chapter 1), a potent AR inhibitor was recently evaluated in a phase III clinical trial in patients with ER^+ tumours has shown a very disappointing 11% overall response rate.[168] This raises the question if estrogen ablation by inhibiting AR alone will be sufficient.

It is known that much of the E1 formed is converted by estrone sulphotransferase (E1-

ST) to the biologically inactive estrone sulphate (E1S), which circulates in blood at a much higher concentration (1 nmol/l)[169] than the other unconjugated E1 or E2 (30-100 pmol/l). The half-life of E1S (10-12 hrs) in blood is considerably longer than that of E1 (20-30 min).[170] The concentration of E1S in breast tissues is also higher than E1 or E2.[171] Therefore, it is likely that E1S in plasma and tissues act as a reservoir for the formation of unconjugated estrogens after it has been hydrolysed by E1-STS.[172] The activity of this enzyme is much higher than that for AR in both normal and malignant breast tissues. Also, the formation of E1 in breast tumours *via* the STS pathway provides at least 10 times as much E1 as that originates from the AR pathway. This constitutes the major source of breast tumour estrogens,[173] accounting for the high concentrations of estrogens detected in such tissues (Figure 2.2).

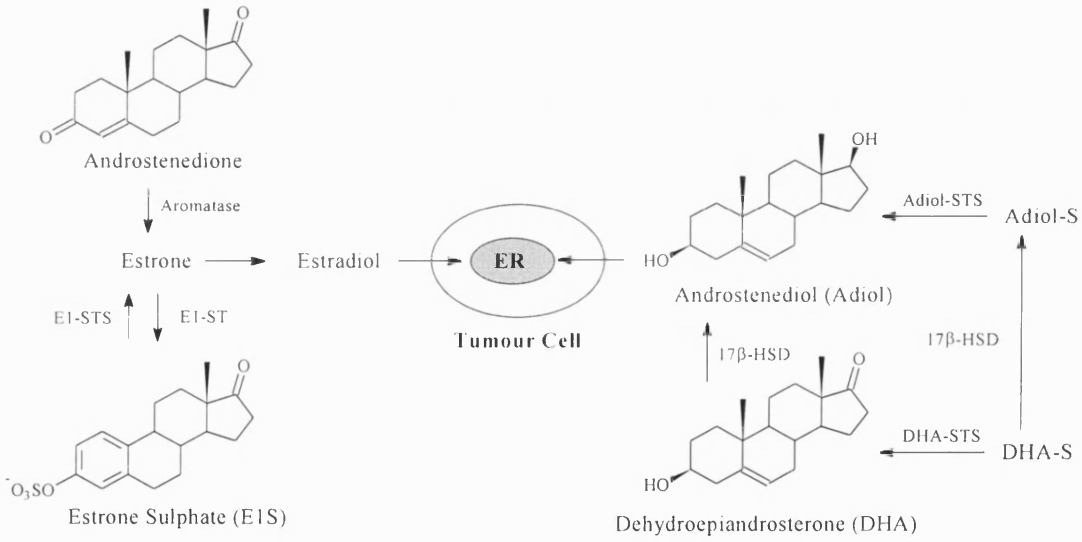


Figure 2.2 : The origins of estrogenic steroids in postmenopausal women

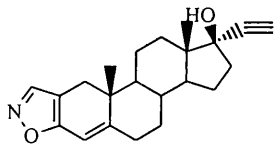
In postmenopausal women it has now been recognised that another major circulating steroid, androstenediol (Adiol) has estrogenic properties. Although Adiol is an androgen, it can bind to the ER and can stimulate the growth of ER^+ breast cancer cells and also carcinogen-induced mammary tumours in the rat.[174,175] There is now strong evidence to suggest that Adiol may be of even greater importance as a promoter of

breast tumour growth.[174] Therefore, stimulation of the AR alone is insufficient to destroy all estrogenic stimuli.

About 90% of Adiol produced in postmenopausal women originate from dehydroepiandrosterone sulphate (DHA-S), which is secreted in large amounts by the adrenal cortex. Two pathways can be envisaged (Figure 2.2). First, DHA-S is hydrolysed to DHA by DHA-sulphatase (DHA-STS), with subsequent metabolism of DHA to Adiol. Alternatively, DHA-S is converted to androstenediol sulphate (Adiol-S) with subsequent hydrolysis to Adiol. Although the affinity of Adiol for ER is lower than that of E2, plasma Adiol concentrations are 100-times higher than those of E2, adding further evidence for DHA-S being the major source of plasma Adiol.[176,177] Therefore, estrogenic stimulation of hormone dependent breast carcinoma can be reduced not only by inhibiting the hydrolysis of E1S but also the production of Adiol from DHA-S. Since there is strong evidence to suggest that E1-STS and DHA-STS are the same enzyme,[162] inhibitors of STS are potential agents for the treatment of HDBCs when used alone or in conjunction with an AR inhibitor.

2.3 Development of steroid sulphatase inhibitors

Among all the identified functions of STS enzymes, regulation of estrogen synthesis in hormone-dependent tumours has been the main one for the development of potent STS inhibitors. Danazol **(2-1)** (an isoxazole derivative of 17β-ethinyltestosterone) (Figure 2.3), which was originally synthesised by Carlstrom[178] as an inhibitor of the conversion of E1 to the more potent E2 by the 17β-HSD, was one of the first synthetic compounds shown to be able to inhibit STS activity weakly in human liver and breast tumour tissues (62% at 10 μM in MCF-7 cells).[179]



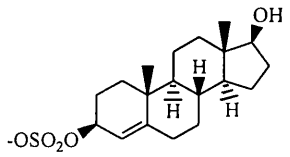
Danazol

2-1

Figure 2.3 : Structure of Danazol

Following the activity observed for Danazol, several steroidal and non-steroidal drugs currently used clinically for endocrine disorders were tested for their ability to inhibit STS enzyme. Antiestrogens,[180] such as tamoxifen and 4-hydroxytamoxifen, and progestogens and their derivatives[181] such as demegestone, chlormadinone acetate, medroxyprogeterone and promegestone were found to be weak inhibitors of STS. These findings and the recognition of the pivotal role of STS enzyme in HDBC have encouraged the synthesis and development of other biologically active and potent inhibitors.

Initial studies in developing STS inhibitors revealed that steroid sulphates could not be ideal candidates for sulphatase inhibition and a greater degree of inhibition was observed with androstenediol-3-sulphate **(2-2)**, which had a K_i value of 2 μM[182] (Figure 2.4). However, a major disadvantage with steroid sulphates is that they also act as substrates for the enzyme, though active *in vitro*, their sulphate group is liable to be hydrolysed *in vivo* releasing the biologically active unconjugated steroids as a consequence.[183]



Androstendiol-3-sulphate

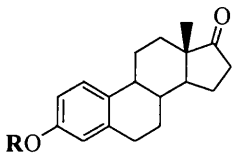
2-2

Figure 2.4 : Structure of androstenediol-3-sulphate

A series of estrone-3-sulphonyl derivatives **(2-3)** was reported to inhibit STS enzyme with the sulphonyl chloride derivative being the best inhibitor achieving a 65% inhibition at 60 μM in placental microsomes[184] (Figure 2.5). Although **2-3** could have inactivated the enzyme *via* sulphonylation, preliminary results showed that other derivatives are only weak competitive inhibitors in this series.[184,185] Cox *et al* in an attempt to generate antisera that are reactive towards E1S has observed that the antibodies, which recognise estrone-3-*O*-methylthiophosphonate (E1-MTP) **(2-4)**, also

recognise E1S[186] (Figure 2.5). When E1-MTP was incubated with liver or placental tissues, E1-MTP was found to be resistant to metabolism, whereas at least 50% of E1S incubated in a similar manner, was converted to E1. This observation suggested that E1-MTP was not an alternative substrate for STS. Since it structurally mimics E1S and its MTP moiety acts as a sulphate group surrogate, and hence E1-MTP is a competitive inhibitor.[187] E1-MTP was found to be 14 times more potent than Danazol in inhibiting E1-STS activity in intact MCF-7 breast cancer cell with a K_i value of 37.5 μM (IC_{50} = 43 μM in placental microsomes).[188]

In vivo, E1-MTP was found to lower the plasma concentration of E2 in treated rats by at least 50% suggesting that E1-MTP is an active competitive inhibitor.[188] When tissues are isolated and homogenised, the inhibitor concentration may be reduced at the enzyme active site. Thus it is quite difficult to demonstrate inhibition in tissues obtained from animals treated with E1-MTP. However, some evidence was obtained to show inhibition *in vivo*. Plasma E2 concentrations were measured in samples obtained from treated (1 mg/Kg per day for 7 days s.c.) and untreated animals, which showed that the plasma E2 concentration was about 50% lower in treated animals, suggesting that E1-MTP is active *in vivo*.

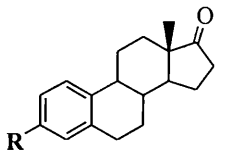


R		
SO_2Cl	= Estrone-3-sulphonyl chloride	**2-3**
PS(Me)OH	= Estrone-3-*O*-methylthiophosphonate (E1-MTP)	**2-4**

Figure 2.5 : Structures of the sulphate derivatives of estrone

Since this finding, other E1S analogues with sulphate surrogates, such as, phosphates[176,189,190] **(2-5)**, phosphonates[191] **(2-6)**, sulphonates[192] **(2-7)**, estrone-3-*O*-methyl sulphonate[189] **(2-8)**, estrone-3-methylene sulphonate[176] **(2-9)**,

thiophosphonates[188,191] **(2-10)** and DHA-3-*O*-phosphate[189] **(2-13)** were synthesised (Figure 2.6).



R		
OPO_3^{2-}	= Estrone-3-*O*-phosphate	**2-5**
$OPO(H)O^-$	= Estrone-3-*O*-*H*-phosphonate	**2-6**
OSO_2^-	= Estrone-3-*O*-sulphonate	**2-7**
OSO_2CH_3	= Estrone-3-*O*-methylsulphonate	**2-8**
$CH_2SO_3^-$	= Estrone methylene sulphonate	**2-9**
$SPO(H)O^-$	= Estrone-3-*S*-phosphonate	**2-10**

Figure 2.6 : Structures of the sulphate derivatives of estrone

Estrone-3-*O*-phosphate[189,176,190] **(2-5)** was found to be relatively potent with a K_i value of 0.89 μM[189]and 0.3 μM[176] at pH 7.0. Anderson and his colleagues synthesised several phosphorylated steroids with geometries closely related to E1S.[189] These phosphate esters are all good reversible inhibitors of STS, binding with an affinity that is better than that of the natural substrate E1S **(2-11)**, but no hydrolysis of the inhibitors was observed. In **2-11**, a putative enzyme active site base acts to deprotonate an incoming water molecule (Figure 2.7 B). The peripheral OH group of the phosphate monoanion of **2-5** is probably well situated to mimic the incoming water molecule and makes a H-bond to the active site base (Figure 2.7 A). Such a combination results in a very strong H-bond and hence a strong inhibition of the enzyme.

Of all the analogues synthesised in this series, the monoanions of steroidal phosphates **2-5** bind to the STS with exceptional affinity by engaging in H-bonding (K_i = 170 nM at pH = 6.0), whereas the singly charged estrone phosphofluoridate **(2-12)** has only H-bond accepting components. Unlike the phosphate ester monoanions, **2-12** cannot donate an H-bond to an enzyme active site amino acid residue. Therefore, it binds with

a much lower affinity than the phosphate ester (K_i = 14.7 μM at pH = 6.0).[189] This observation indicated that phosphates largely exist as monoanions at neutral pH and serve as better STS inhibitors than do sulphates.

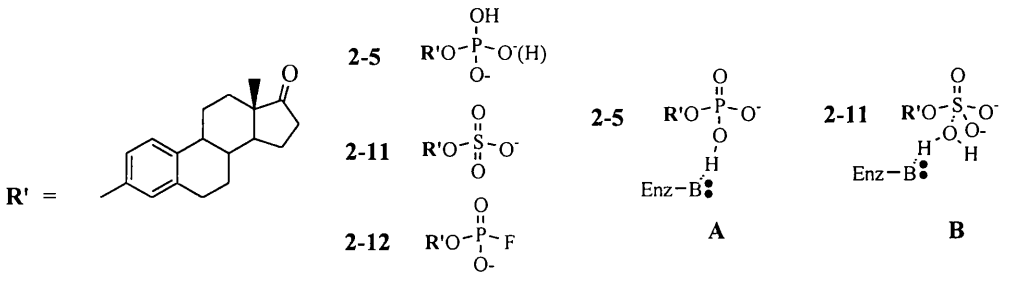
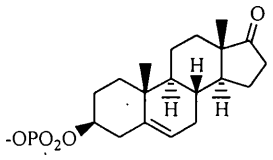


Figure 2.7 : Structures of the phosphate ester derivatives and the mechanisms of enzyme-catalysed reaction **A** and **B**.

Estrone-3-*O*-*H*-phosphonate[189] **(2-6)** showed a 80% E1-STS inhibition at 10 μM in MCF-7 cells.[190] Estrone-3-methylene sulphonate[176] **(2-9)** showed a 100 fold increase in inhibition than E1S indicating that the bridging O atom in E1S is essential for high affinity binding. Estrone-3-*O*-methyl sulphonate **(2-8)** and DHA-3-*O*-phosphate[189] **(2-13)** (Figure 2.8) were found to be relatively weaker inhibitors than E1-3-*O*-phosphate **(2-5)**.



Dehydroepiandrosterone-3-*O*-phosphate

2-13

Figure 2.8 : Structures of dehydroepiandrosterone-3-*O*-phospahate

Other derivatives of steroid sulphate have also been synthesised by various groups around the world and tested for their sulphatase inhibitory properties and some indeed showed interesting activities, which have been tabulated in Table 2.1.

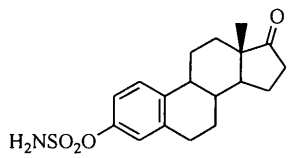
Compounds	% Inhibition (10 μM)
E1-3-*O*-*H*-phosphonate **(2-6)**	80
E1-3-*O*-methylphosphonate **(2-14)**	41
E1-3-*O*-ethylphosphonate **(2-15)**	53
E1-3-*O*-phenylphosphonate **(2-16)**	42
E1-3-*O*-methylsulphonate **(2-8)**	28
E1-3-*O*-ethylsulphonate **(2-17)**	27
E1-3-*O*-butylsulphonate **(2-18)**	17
E1-3-*O*-phenylphosphoramidate **(2-19)**	32

Table 2.1 : Inhibition of E1-STS activity in MCF-7 cells by various derivatives of estrone sulphate.[191,176]

2.4 Development of steroid sulphamates

All the derivatives of E1S synthesised, including E1-MTP, provided with some indication of the type of derivatives, which may be useful as steroid sulphatase inhibitors. The first steroid sulphatase inhibitor discovered with highly potent biological activity was estrone-3-*O*-sulphamate **(2-20)** (EMATE) (IC_{50} in MCF-7 breast cancer cells = 65 pM and 25 nM in human placental microsomes)[187] (Figure 2.9).

EMATE inhibits over 99% of E1-STS activity in intact MCF-7 cells at 0.1 μM and it is an active site-directed inhibitor showing time- and concentration-dependent inactivation of the enzyme. It is also highly potent *in vivo* against rat liver E1-STS and DHA-STS activities, when administered either orally or subcutaneously.



Estrone-3-*O*-sulphamate (EMATE)

2-20

Figure 2.9 : Structure of estrone-3-*O*-sulphamate

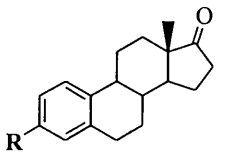
Various analogues of EMATE were synthesised, where the NH_2 protons were methylated and the bridging O atom was replaced with other heteroatoms such as sulphur or nitrogen. The *N*-monomethylated **(2-21)** and *N,N*-dimethylated **(2-22)** derivatives of EMATE, whilst still more potent than E1-MTP, are less potent than EMATE in inhibiting sulphatase activity (79% and 52% inhibition at 0.1 μM respectively in MCF-7 cells).[193] These compounds showed a weak, non-time dependent inhibition of E1-STS.[194] Derivatives such as estrone-3-sulphamide **(2-23)**, estrone-3-*S*-sulphamate **(2-24)** and estrone-3-*S*-(*N,N*-dimethyl)sulphamate **(2-25)** were all found to be weak non-time dependent inhibitors[194] (Figure 2.10). These weak inhibitory activities shown by these heteroatom containing derivatives of EMATE have suggested that the bridging O atom in the sulphamate group might involve in the binding of the inhibitor to the enzyme active site *via* H-bonding and that the leaving group ability of the parent steroid might play an important role in the inactivation of the enzyme.

Woo *et al*[195] acylated the N atom on the sulphamoyl group and also replaced all its protons with large alkyl groups. It was found that the *N*-acylated derivative **(2-26)** is an irreversible inhibitor, but less potent than EMATE, whereas the *N*-benzylated derivative, *N,N*-dibenzyl sulphamate **(2-27)**, 3-*O*-*N*-(piperidino)sulphamate **(2-28)** and the 3-*O*-(*N*-benzoyl)sulphamate **(2-29)** are weakly reversible inhibitors (Figure 2.10).

Extensive structure-activity studies on EMATE and other related compounds have confirmed that the sulphamoyl group attached to an aryl ring is the active pharmacophore required for potent inhibition of steroid sulphatase activity.[196] It has

also been shown that the bridging O atom between the steroid nucleus and the sulphamoyl group is indispensable for the inhibitory activity of EMATE and the NH_2 protons on the sulphamoyl group are also important for E1-STS inhibition.

Even though EMATE is a potent sulphatase inhibitor, it has been shown recently to be highly estrogenic and hence EMATE in principle is unlikely to be developed for use in the clinical treatment of hormone dependent cancers in women. It is about five-times more active than the synthetic estrogen, ethinylestradiol on oral administration to rats.[197] Although the real reason is yet to be elucidated, current evidence suggests that in the body EMATE is acting as a prodrug of estrone. Since the endocrine dependent tumours of the breast and endometrium are sensitive to E1, EMATE is unsuitable for use as an endocrine therapeutic agent.



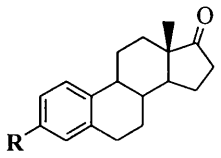
R		
OSO_2NH_2	= Estrone-3-*O*-sulphamate (EMATE)	**2-20**
OSO_2NHMe	= Estrone-3-*O*-(*N*-methyl)sulphamate	**2-21**
OSO_2NMe_2	= Estrone-3-*O*-(*N,N*-dimethyl)sulphamate	**2-22**
$NHSO_2NH_2$	= Estrone-3-sulphamide	**2-23**
SSO_2NH_2	= Estrone-3-*S*-sulphamate	**2-24**
SSO_2NMe_2	= Estrone-3-*S*-(*N,N*-dimethyl)sulphamate	**2-25**
$OSO_2NHCOCH_3$	= Estrone-3-*O*-(*N*-acetyl)sulphamate	**2-26**
$OSO_2N(CH_2Ph)_2$	= Estrone-3-*O*-(*N,N*-dibenzyl)sulphamate	**2-27**
OSO_2N 	= Estrone-3-*O*-*N*-(piperidino)sulphamate	**2-28**
$OSO_2NHCOPh$	= Estrone-3-*O*-(*N*-benzoyl)sulphamate	**2-29**

Figure 2.10 : Derivatives of EMATE

Although, EMATE has been developed primarily for use in the treatment of HDBC, it has also been shown to have memory-enhancing effects in rats by inhibiting the conversion of dehydroepiandrosterone sulphate (DHA-S) to dehydroepiandrosterone

(DHA) suggesting an application in Alzheimer's disease.[198] It's super-estrogenicity can be further explored in therapeutic areas such as oral contraception and hormone replacement therapy (HRT).[199,200] In addition, EMATE might have a role in regulating the immune response since studies in mice have suggested a role of DHA-STS activity in regulation of part of the immune response[201,202] and such a relationship may also be found in humans. Whilst agents clinically used for Alzheimer's disease and HRT targets elderly patients, STS inhibitors as regulators of immune response may have to be administered from an early age. It is thus anticipated that a potent orally active irreversible non-steroidal E1-STS inhibitor, which is metabolically stable and devoid of estrogenic activity will have much wider clinical applications than their steroidal counterparts.

Other estrone derivatives such as 3-aminoestrone **(2-30)** and 3-thioestrone **(2-31)** were synthesised by Selcer and Li in order to investigate whether these STS inhibitors are converted in to any estrogenic metabolites *in vivo*[203] (Figure 2.11). When tested in ovariectomised rats on growth of the rat uterus, these compounds were found to be well tolerated *in vivo* at up to 100 μg/day and had no effect on uterine growth, indicating they are non-estrogenic. Also, they were found to be non-antiestrogenic, since they did not block the ability of E2 to stimulate uterine growth.



R = NH_2 **2-30**
SH **2-31**

Figure 2.11 : Derivatives of estrone

A large number of A and D ring modified EMATE derivatives were also synthesised and tested for E1-STS activity since it has been shown previously that C-2 and/or C-4 substituted and D-ring modified estrogens are less estrogenic than their parent compounds.[204,205] Of all the A-ring modified analogues synthesised, the 2/4-allyl-EMATEs **(2-32, 2-33, 2-34)** are more potent than the 2/4-alkyl analogues **(2-35, 2-36, 2-37)**, but they were all less potent than EMATE in placental microsome

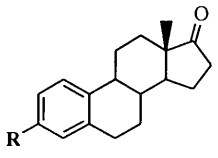
preparations.[206] In contrast, the 4-nitro EMATE **(2-39)** was more potent than its 2-nitro analogue **(2-38)** and also found to be 5 times more potent than EMATE *in vivo* (Table 2.2).

Compounds	IC_{50} (μM)
EMATE **(2-20)**	0.004
2-Allyl EMATE **(2-32)**	2.5
4-Allyl EMATE **(2-33)**	9.0
2,4-Diallyl EMATE **(2-34)**	>100
2-*n*-Propyl EMATE **(2-35)**	29
4-*n*-Propyl EMATE **(2-36)**	> 100
2,4-Di-*n*-propyl EMATE **(2-37)**	>100
2-Nitro EMATE **(2-38)**	0.07
4-Nitro EMATE **(2-39)**	0.0008
2-MeOEMATE **(2-40)**	0.003
2-EthylEMATE **(2-41)**	2.4
2-BromoEMATE **(2-42)**	12.6×10^{-6}
2-IodoEMATE **(2-43)**	0.0009

Table 2.2 : IC_{50} values of some A-ring modified EMATEs in placental microsomes

Earlier studies have also shown that an oxygen atom or at least a sterically or electronically similar link between the steroid ring and sulphonate moiety is essential for higher affinity towards the STS enzyme. Selcer *et al* designed and synthesized a series of estrone-3-amino derivatives as potential E1-STS inhibitors[207] and tested their inhibitory potential using human placental microsomes, which contain a substantial amount of E1-STS activity. Several compounds (Figure 2.12) in the series significantly inhibited E1-STS activity of the human placental microsomes at 10 μM. The IC_{50} of these compounds ranged from 8.7 to 14.6 μM, the most potent being compound **2-45**. They also tested the ability of these amino derivatives to inhibit growth of the estrogen-

dependent MCF-7 breast cancer cell line, where they showed substantial proliferation in the presence of 100 nM E1S in estrogen-free media, indicating that the cells were capable of converting E1S into E1.[207]

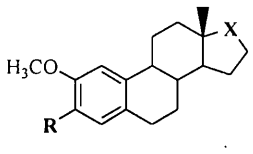


R =	$NHSO_2CF_3$	**2-44**
	$N(CF_3SO_2)_2$	**2-45**
	$NHCOCF_3$	**2-46**
	$NHCONH_2$	**2-47**

Figure 2.12 : Amino derivatives of estrone

There is currently a considerable interests in the role that 2-methoxyestrone (2-MeOE1) **(2-48)** and its endogenous estrogen metabolite, 2-methoxyestradiol (2-MeOE2) **(2-49)** may have for development as a therapy for ER^+ and ER^- mammary tumours (Figure 2.13). 2-MeOE2 has been shown to inhibit *in vitro* angiogenesis and the growth of MDA-MB-435 ER- breast cancer cells *in vivo,* which may be due to its ability to inhibit tumour-induced angiogenesis. (See Chapter 6)

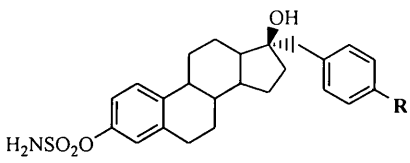
2-Methoxyestrone-3-*O*-sulphamate (2-MeOEMATE) **(2-40)** and 2-methoxyestradiol-3-*O*-sulphamate (2-MeOE2MATE) **(2-50)** synthesised in our group were found to be potent non-estrogenic E1-STS inhibitors (Figure 2.13). 2-MeOEMATE was equipotent with EMATE as a steroid sulphatase inhibitor, but was found to posses potent anti-mitotic properties, suggesting that it will be suitable not only for ER^+/ER^- breast cancers, but also for other types of cancers.[208] In addition, other 2-substituted compounds, such as 2-ethyl **(2-41)**, 2-bromo **(2-42)** and 2-iodo **(2-43)** EMATEs were also found to be potent inhibitors of E1-STS and also inhibited angiogenesis (Unpublished results) (Table 2.2). The most potent inhibitor being the 2-bromo analogue about 317 times more active than EMATE and the 2-iodo derivative was about 4 times more potent.



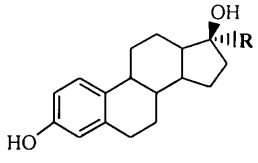
R	X		
OH	C=O	**2-48**	**2-MeOE1**
OH	CHOH	**2-49**	**2-MeOE2**
OO_2SNH_2	C=O	**2-40**	**2-MeOEMATE**
OO_2SNH_2	CHOH	**2-50**	**2-MeOE2EMATE**

Figure 2.13 : 2-Methoxy derivatives of estrone and EMATE

Apart from A ring modified derivatives, modifications at other positions of the estrone molecule have also been carried out. The estradiol derivatives with substituents at the 17α-position, synthesised by Ciabanu *et al* were found to be potent STS inhibitors *in vitro*.[209] Hence, 17α-benzylestradiol **(2-51)** and (4'-*tert*-butylbenzyl)estradiol **(2-52)** inhibit STS activity with IC_{50} values of 0.39 nM and 0.15 nM respectively, probably due to interactions of their 17α-substituents with a hydrophobic pocket around the region of the enzyme active site (Figure 2.14). Unlike EMATE, which is an active site directed irreversible inhibitor of E1-STS these sulphamoylated derivatives of E2 are only reversible inhibitors. Further analogues in the series with an *n*-octyl group[210] **(2-53)**, alkyl amide **(2-54)** and alkyn amide side chain **(2-55)** at the 17α position of estradiol were also found to be potent STS inhibitors with IC_{50} values of 0.44 μM, 0.08 μM and 0.35 μM respectively.[211] The increase in potency observed may be due to effective binding at the enzyme active site as a result of an increase in hydrophobicity. The amide group is expected to block the activation of the ER effectively.

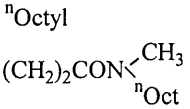


R = H **2-51**
$C(CH_3)_3$ **2-52**

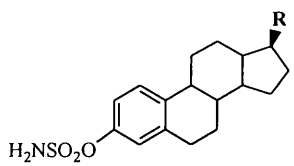


R = ^{n}Octyl **2-53**

2-54



2-55



R = $-CONH(CH_2)_6CH_3$ **2-56**
$-NHCO(CH_2)_6CH_3$ **2-57**

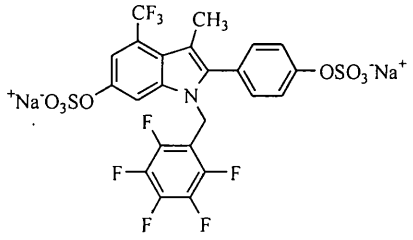
Figure 2.14 : 17-Substituted derivatives of estrone and EMATE

17β- substitued alkyl carbamoyl **(2-56)** and *N*-alkanoyl derivatives of EMATE **(2-57)** synthesised by Li *et al* contains all the required structural features for a potent STS inhibitor were found to inhibit E1-STS with IC_{50} values in the range of 0.5 nM (Figure 2.14). These compounds were also shown to be devoid of estrogenicity when compared with EMATE.[212]

2.5 Development of non-steroidal sulphatase inhibitors

With the drawback of EMATE being a 'super-estrogen', and the potential endocrinologically active metabolites of steroidal inhibitors, much focus over the years has been on the development of non-steroidal sulphatase inhibitors which themselves or through their metabolites are unlikely to possess any undesirable endocrinological effects.

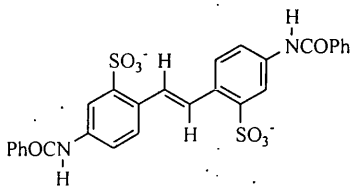
The first series of non-steroidal steroid sulphatase inhibitors was based upon 5-acetoxy-3-(4-acetoxyphenyl)-1-ethyl-3-methylindole, which was originally designed as a new class of mammary tumour inhibitors. Birnbock and von Angerer synthesised mono- and bis- sulphate derivatives of 2-phenylindoles and one of the compounds 3-methyl-1-pentafluorophenylmethyl-6-sulphooxy-2-(4-sulphooxyphenyl)-4-trifluoro methylindole, disodium salt **(2-58)** was found to be a competitive sulphatase inhibitor with an IC_{50} value of 80 μM in homogenate of calf uterus (Figure 2.15). However, this sulphate was shown to be hydrolysed to free phenols upon incubation with the steroid sulphatase.[213]



2-58

Figure 2.15 : Structure of 3-Methyl-1-pentafluorophenylmethyl-6-sulphooxy-2-(4-sulfooxyphenyl)-4-trifluoromethylindole, disodium salt

Dibbelt and Kuss reported the sulphatase inhibitory activities of several 4,4'-substituted stilbene-2,2'-disulphonate derivatives of which 4,4'-dibenzoylaminostilbene-2,2'-disulphonate **(2-59)** was the most potent inhibitor with a K_i of 3 μM[154] (Figure 2.16).



2-59

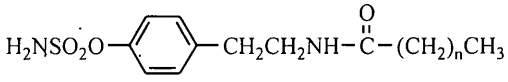
Figure 2.16 : Structure of 4,4'-dibenzoylaminostilbene-2, 2'-disulphonate

The first series of non-steroidal inhibitors synthesised in our group was based upon tetrahydronaphth-2-ol (THN) and diethylstilbestrol (DES) derivatives, which are presumably A/B- and A/D- ring mimics of EMATE, respectively (Figure 2.17). Three sulphamate derivatives of THN were synthesised of which the *N,N*-dimethylsulphamate **(2-63)** derivative was inactive *in vitro* when tested at 10 μM. The THN-sulphamate **(2-61)** was the best inhibitor in the series with an inhibitory activity of 15-79% over the 0.1-10 μM range in intact MCF-7 cells. Incubating MCF-7 cells with **2-61**, as previously used to investigate the nature of EMATE inhibition, revealed that E1-STS activity only inhibited by 22% compared with the activity in untreated cells. This suggests that **2-61** may be acting as a weak irreversible inhibitor.[214] The bis-sulphamate derivatives of DES were found to be considerably more potent inhibitors than the THN derivatives. Like EMATE, DES bis-sulphamates appears to act as irreversible inhibitors. DES-bis-*N,N*-dimethyl sulphamate **(2-65)** inhibited activity by more than 90% at all concentrations tested (1-10 μM).

X		Y	
2-60	OH	**2-64**	OSO_2NH_2
2-61	OSO_2NH_2	**2-65**	OSO_2NMe_2
2-62	OSO_2NHMe		
2-63	OSO_2NMe_2		
Tetrahydronaphth-2-ol Derivatives		**Diethylstilbestrol Derivatives**	

Figure 2.17 : Derivatives of tetrahydronaphth-2-ol and diethylstilbestrol

Li *et al* developed a series of (*p-O*-sulphamoyl)-*N*-alkanoyl tyramines **(2-66)**, which were found to be potent E1-STS inhibitors in human placental microsomes[215] (Figure 2.18). It was argued that the phenyl sulphamate portion of the inhibitors mimics the A ring whereas the *N*-alkanoyl group provides a hydrophobic bulk mimicking the B, C and D rings and the carbonyl group mimics the C-17 C=O of EMATE.



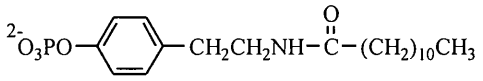
2-66

Figure 2.18 : Structure of (*p-O*-sulphamoyl)-*N*-alkanoyl tyramines

Li *et al* observed an increase in activity when they increased the alkanoyl chain length from n = 5 to 13. This is possibly due to increased hydrophobic interactions between the alkanoyl chain and the hydrophobic binding site of the enzyme. STS is a membrane bound enzyme therefore it has also been argued that these hydrophobic substituents might act as membrane inserters delivering the inhibitors to the enzyme. [215] The best inhibitor in this series, (*p-O*-sulphamoyl)-*N*-tetradecanoyl tyramine **(2-66)** with an alkanoyl chain length of fourteen carbons was found to have an IC_{50} of 55.8 nM in

human placental microsomes[215] (Figure 2.18). Based on this observation their recent series of compounds (*p*-*O*-sulphamoyl)-*N*-alkanoylphenylalkylamines was shown to be potentially active E1-STS inhibitors. The best compound in this series had an IC_{50} of 72 nM in human placental microsomes.[216]

Anderson *et al* have also reported a similar observation on extensive interaction between the enzyme and the hydrophobic side chain, by synthesising various non-steroidal phosphate esters, such as aryl phosphates, tetrahydronaphthyl phosphates and phosphorylated tyramines.[217]



2-67

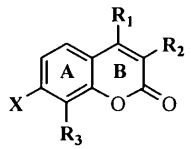
Figure 2.19 : Structure of *n*-lauroyl tyramine phosphate

The best non-steroidal inhibitor in these series of compounds was found to be *n*-lauroyl tyramine phosphate **(2-67)** which inhibited E1-STS with a K_i of 520 nM at pH 7.0[217] (Figure 2.19). The increased hydrophobicity by the long alkyl chain enhances the partitioning in to a non-polar environment and presents the sulphatase enzyme with a locally higher inhibitor concentration, hence acting as membrane inserters.

In pursuit of alternative non-steroidal mimics of EMATE after the modest success with the THN sulphamate **(2-61)** (Figure 2.17), the monohydroxylated coumarin structure was chosen by our group for modification, whose ring system is ubiquitous in nature and in many pharmaceuticals. The core coumarin, 7-hydroxycoumarin might mimic the A/B-ring of EMATE and structurally it differs from THN at the B ring by possessing an α,β-unsaturated lactone in place of a saturated cyclic hydrocarbon. We reasoned that the extended conjugation of such coumarin structures over THN should enhance the overall activity by lowering the relative pK_a of the leaving phenol released during enzyme inactivation. Also, having alkyl chain lengths increase the hydrophobicity of

the compounds and hence enhances the interaction with the enzyme-binding site.[214]

7-(Sulphooxy)-4-methylcoumarin **(2-69)** was the first compound synthesised to examine if coumarin sulphates are substrates for E1-STS. In a placental microsomal preparation, the hydrolysis of **2-69** by E1-STS was completely abolished when EMATE was included in the reaction mixture, indicating that the coumarin sulphate is a substrate for the sulphatase enzyme. Therefore the sulphamate derivatives of coumarin are expected to act as sulphatase inhibitors; hence a series of coumarin sulphamates were prepared (Table 2.3).



	X	R_1	R_2	R_3
2-68	OH	H	H	H
2-69	OSO_3	CH_3	H	H
2-70	OSO_2NH_2	H	H	H
2-71	OSO_2NH_2	CH_3	H	H
2-72	OSO_2NH_2	CH_3	CH_3	CH_3
2-73	OSO_2NH_2	CF_3	H	H

Table 2.3 : Some of the initial coumarin analogues synthesised

Of all the initial compounds synthesised, 4-methylcoumarin-7-*O*-sulphamate (COUMATE) **(2-71)** (Table 2.3) was found to be the most active with an IC_{50} value of 380 nM in intact MCF-7 cells, exhibiting similar inhibitory profiles to those of EMATE. Although it is less potent and its duration of inhibition *in vivo* is much shorter,[214] like EMATE, **2-71** is orally active and acts in a time- and concentration-dependent manner, suggesting that it may have a similar mechanism of action.[196]

However, in contrast to EMATE, **2-71** is non-estrogenic as shown by its lack of stimulatory effect on the growth of uterus in ovariectomised rats.[218] The structure-activity relationships for **2-71** were studied recently, which have shown, *inter alia*, that the coumarin ring system is pivotal for its inhibitory activities.[196]

		% Inhibition ± S.D. of E1-STS activity in					
		placental microsomes			**MCF-7 breast cancer cells**		
		10 μM	**1 μM**	**0.1 μM**	**10 μM**	**1 μM**	**0.1 μM**
2-70	C3-H, C4-H (COUMATE)	78 ± 1	*	*	*	*	*
2-71	C3-H, C4-CH_3	93 ± 1	63 ± 1	< 10	93 ± 1	86 ± 1	43 ± 1
2-74	C3-CH_3, C4- CH_3,	97 ± 4	88 ± 2	35 ± 1	98 ± 1	95 ± 1	83 ± 1
2-75	C3-H, C4-CH_3CH_2	> 99	88 ± 1	35 ± 1	92 ± 1	90 ± 1	77 ± 1
2-76	C3-H, C4-$CH_3(CH_2)_2$	96 ± 1	94 ± 1	42 ± 1	94 ± 1	89 ± 1	81 ± 1
2-77	C3-CH_3CH_2, C4-CH_3,	> 99	96 ± 1	57 ± 1	99 ± 1	97 ± 1	92 ± 1
2-78	C3-$CH_3(CH_2)_2$, C4-CH_3,	> 99	97 ± 1	83 ± 1	99 ± 1	98 ± 1	94 ± 1
2-79	C3-$CH_3(CH_2)_3$, C4-CH_3,	>99	>99	90 ± 1	99 ± 1	99 ± 1	97 ± 1

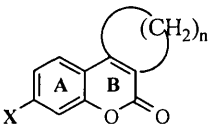
Table 2.4 : Inhibition of E1-STS activity by 4-alkylated and 3-alkyl-4-methyl coumarin sulphamates. Compounds **2-70**, **2-71** and **2-74** are included for comparison.

One of the analogues of **2-71**, 3,4-dimethylcoumarin-7-*O*-sulphamate **(2-74)** (Table 2.4) was found to be about 12 times more potent than **2-71** (IC_{50} = 30 nM in MCF-7 cells, c.f. IC_{50} = 380 nM for **2-71**). It is evident that the higher potency of **2-74** in comparison with **2-71** is attributed to its stronger binding to the enzyme active site as a result of hydrophobic interactions with neighbouring amino acids provided by the methyl group at the 3-position. Therefore, it is reasonable to expect that the analogues of **2-71** and **2-74** with substituents of increased hydrophobicity at the C-3 and/or C-4 positions would be even more effective sulphatase inhibitors. Such hydrophobicity-activity relationship is also implicated by Li and Anderson *et al* in their work on (*p-O-*

sulphamoyl)-*N*-alkanoyl tyramines[215] and n-lauryl tyramine phosphate respectively.[217]

In order to test out such a prediction, several other coumarin sulphamates were synthesised (Table 2.4). As shown in table 2.4, all these coumarin sulphamates are potent E1-STS inhibitors with the highest potency being observed for the 3-(*n*-butyl)-4-methyl analogue **2-79** in MCF-7 cells and placental microsomes.[219] **2-75** is equipotent with **2-74** in placental microsomes at 0.1 μM, while, **2-75** and **2-76** are less potent than **2-74** in MCF-7 cells. Compounds **2-77**, **2-78** and **2-79** are all showed an increase in potency with chain length indicating that chain extension at the C-3 position is more effective than at C-4 position.

While the hydrophobicity of coumarin sulphamates is significantly increased by introducing linear alkyl chains at the C-3 and/or C-4 positions of the coumarin ring, it is reasonable to expect a similar effect could be achieved by incorporating an extra ring structure at the C3-C4 junctions of the coumarin ring. A series of tricyclic coumarin sulphamates, whose third ring differ in size, were hence synthesised (Table 2.5).



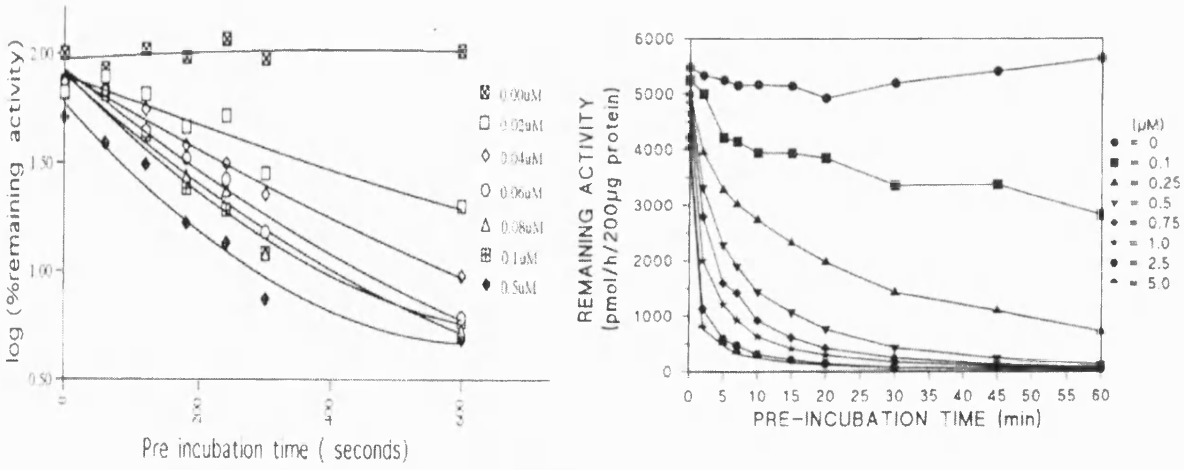
X = OSO_2NH_2

	Ring size	N	% Inhibition ± S.D. of E1-STS activity in human placental microsomes			
			0.01 μM	0.1 μM	1 μM	IC_{50} (μM)
2-80	665 COUMATE	3	< 10	37 ± 1	91 ± 2	200
2-81	666 COUMATE	4	< 10	63 ± 1	93 ± 1	70
2-82	667 COUMATE	5	48 ± 2	91 ± 1	99 ± 1	8
2-83	668 COUMATE	6	17 ± 2	89 ± 1	99 ± 2	30
2-84	6613 COUMATE	11	< 10	17 ± 2	77 ± 2	75

Table 2.5 : Inhibition of E1-STS activity in placental microsomes by tricyclic coumarin sulphamates at various concentrations.[220]

As predicted, this series of tricyclic coumarin sulphamates were found to be highly effective STS inhibitors with the highest potency being observed for compound **2-82** which has a 7-membered third ring (667 COUMATE) (Table 2.5). The IC_{50} value for 667 COUMATE was found to be 8 nM[220] suggesting that this inhibitor is about three-times and 100-times more potent than EMATE (IC_{50} = 25 nM) and COUMATE (IC_{50} = 800 nM) respectively. However, a jump from a cycloheptyl to a cyclotridecanyl third ring was found to be detrimental as shown by the poorer inhibitory activity of compound **2-84**.[220]

The time- and concentration- dependent inactivation of the E1-STS activity by 667 COUMATE **(2-82)** was found to be similar to those of EMATE.[221,196] As shown in figure 2.20 A,[219] the inhibition by 667 COUMATE is biphasic, indicating that the inhibitor shares a similar mechanism of action to that proposed for EMATE (Figure 2.20 B) which, we have postulated that it acts *via* irreversible sulphamoylation of one or more essential amino acid residues in the enzyme active site.[196]



A **B**

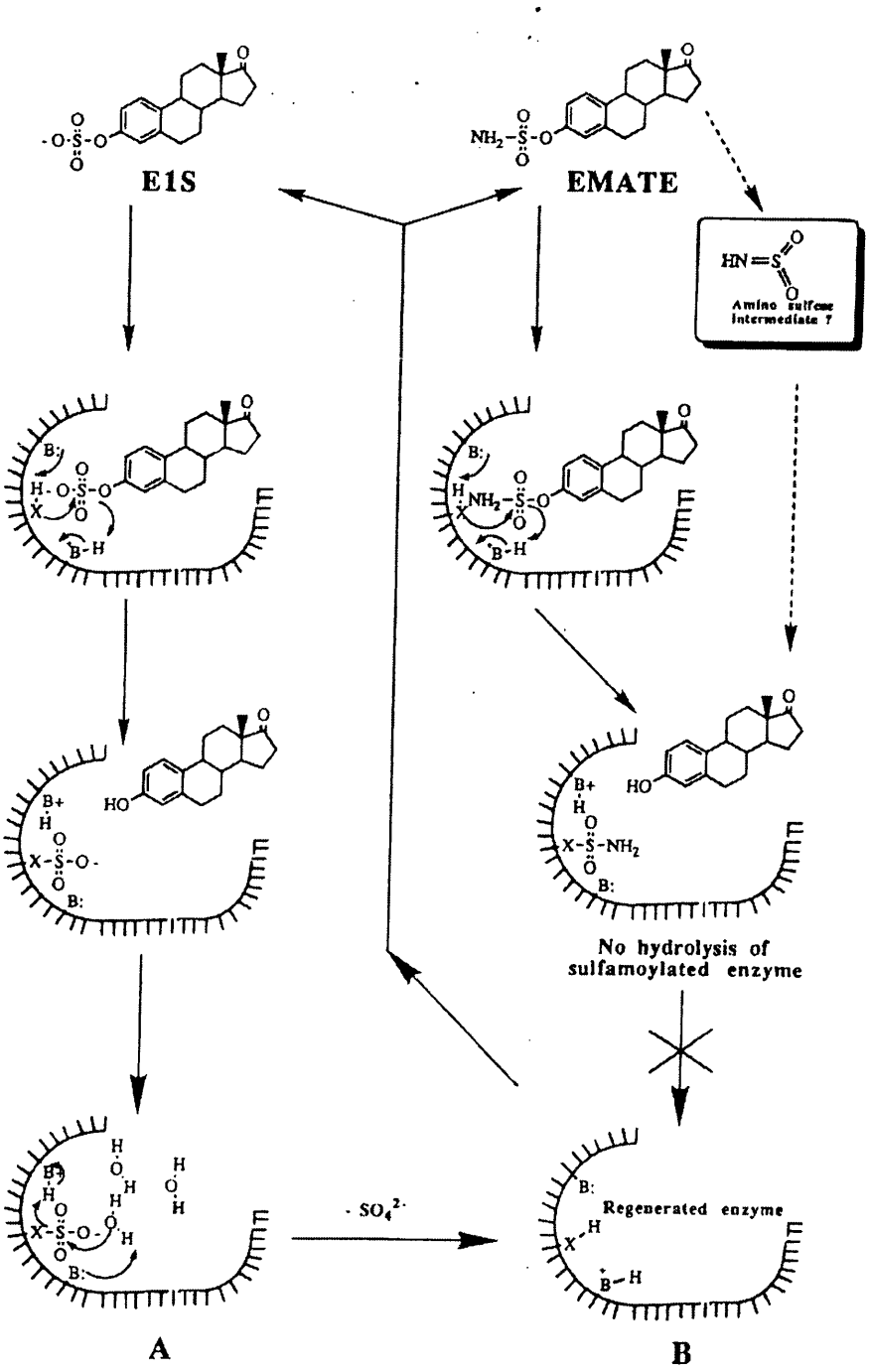
Figure 2.20 : Time- and concentration- dependent inactivation of E1-STS activity in placental microsomes by 667 COUMATE **(A)**[219] and EMATE **(B)**.[196]

The estrogenicity of 667 COUMATE was assessed *in vitro* using ER^+ MCF-7 cell lines

in vitro, which usually responds to low concentrations of E2 and stimulates cell growth by 150% compared with the controls at 10^{-9} M. With 667 COUMATE, the cells showed no significant effects on the proliferation of MCF-7 cells over a range of 10^{-11} to 10^{-5} M, strongly indicating that 667 COUMATE is devoid of estrogenicity.[208]

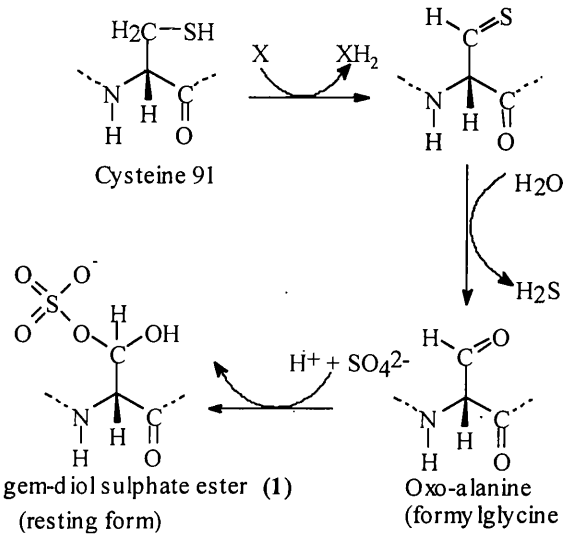
2.6 Initially proposed mechanism of steroid sulphatase inhibition by EMATE

The mechanism of aryl sulphate hydrolysis was first proposed by Purohit *et al* and they postulated that the mechanism may be analogous to the proposed mechanism of sulphuryl transfer by aryl steroid sulphotransferase.[222,223] They proposed that the mechanism involves a direct nucleophilic displacement of E1 from E1S, either directly by water (A) or by a two step mechanism, which involves hydrolysis of a sulphated enzyme intermediate to regenerate the active enzyme (Scheme 2.1). EMATE involves the essential amino acid residue, which is normally sulphated during catalysis or a neighbouring residue becomes irreversibly sulphamoylated, either by direct nucleophilic attack at the sulphur atom of EMATE by the enzyme or by the formation of a reactive aminosulphene intermediate by proton abstraction at nitrogen and subsequent loss of estrone, followed by rapid sulphamoylation of the enzyme (B).[224]



Scheme 2.1 : Initially proposed mechanism of estrone sulphate hydrolysis by steroid steroid sulphatase and enzyme inactivation by EMATE. (A) Direct nucleophilic displacement of E1 from the sulphate group of E1S. (B) Involvement of either the essential amino acid residue, or a neighbouring residue becomes irreversibly sulphamoylated.

The catalytic sites of lysosomal sulphatase and human arylsulphatase A, identified from the crystal structures have now suggested new mechanisms for sulphatase ester hydrolysis.[219] In all the different sulphatases whose structures have been resolved so far, the active site amino acid residues are conserved. Bond *et al* showed that in ASB, hydrolysis takes place *via* post-translational modification of a specific-active site cysteine residue to oxo-alanine (Scheme 2.2).



Scheme 2.2 : The post translational modification of the essential active site cysteine residue in *N*-acetylgalactosamine-4-sulphatase (ASB) (Adapted from Bond *et al*[159])

2.7 Mechanism of sulphate cleavage by sulphatase enzymes

To understand the catalytic mechanism of sulphatases, Bond *et al* determined the crystal structure of ASB.[159] The active site of ASB was readily located by the presence of a cluster of conserved residues surrounding the post-translationally modified essential amino acid cysteine residue (Cys91), which is conserved by a sulphate adduct of FGly [-CH(OH)-SO_3-]. The conserved residues that line the active site pocket are Asp53, Asp54, Cys91, Pro93, Ser94, Arg95, Lys145, His147, His242, Asp300, and Lys318 (Figure 2.21). The sulphate is bonded to a metal ion, which has been identified as calcium. It also has been identified that the electron density around the sulphate is due to the covalently bonded gem-diol sulphate ester **1** (Scheme 2.2). The sulphate

ester observed in the crystals may have formed by the addition of the sulphate to the aldehyde resulting from Cys91 modification. Schmidt *et al* proposed that this cysteine residue in sulphatase undergoes a conversion to oxo-alanine and the free aldehyde is stablised as enol or linked to an amino or hydroxyl group of the polypeptide forming a Schiff's base or a hemi-acetal respectively.[225] The presence of the aldehyde group in the residue was demonstrated by reduction of the enzyme with sodium borohydride under conditions specific for aldehyde reduction, and identification of a resultant serine residue in the sequence in place of the encoded cysteine.[225]

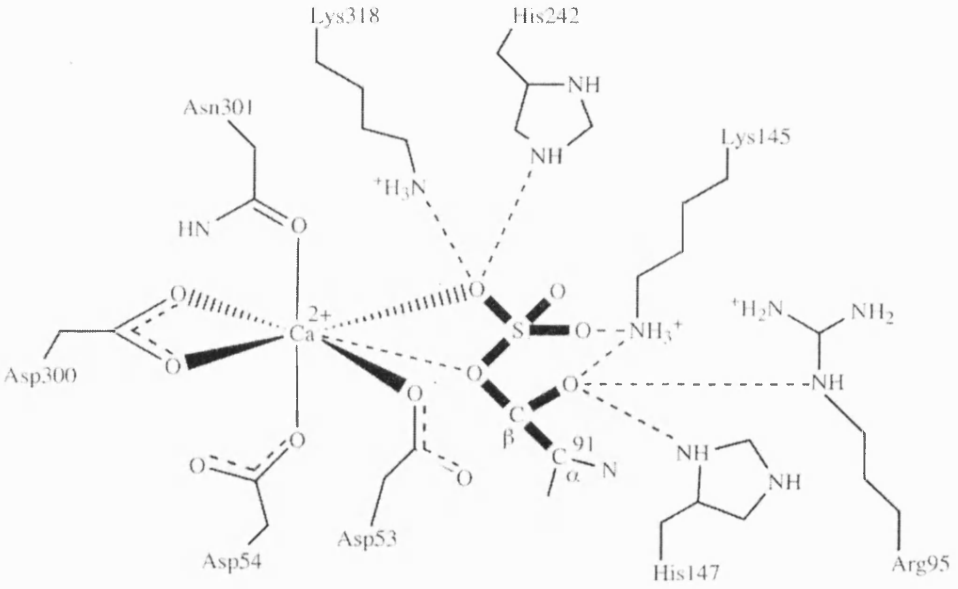
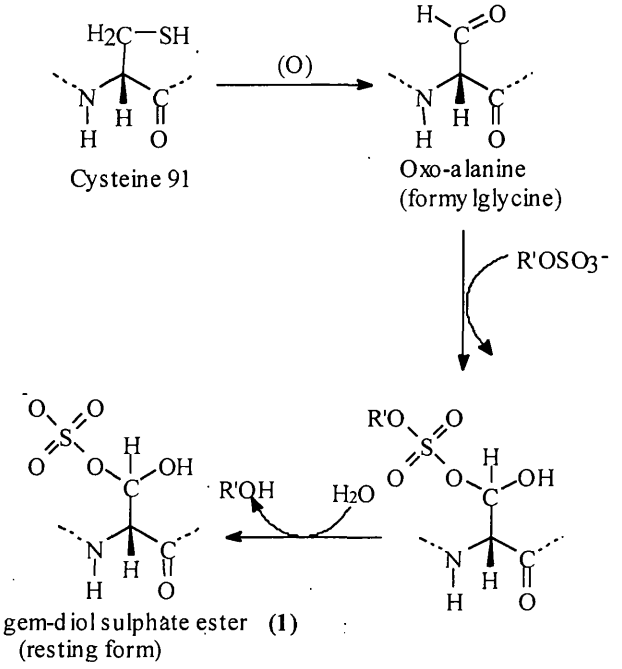


Figure 2.21 : Sketch view of the catalytic site of ASB showing H-bonding (dashed lines) that stablises the sulphate ester. The seven-coordinated Ca^{2+} metal ion is on the left. A salt bridge interaction between Lys145 and the carboxyl O atom of Asp53, which is not coordinated to the metal ion and a number of charges and double bonds are omitted for clarity. (Adapted from Bond *et al*[159])

During the hydrolysis cycle of the substrate ester by ASB, the sulphate ester **1** may be released possibly by an attack by a nucleophile such as water or it could be relatively weakly bound to the enzyme and in equilibrium with the free aldehyde form. When the

substrate of ASB, glycosaminoglycan binds to the enzyme, the sulphate group covalently binds to residue 91. A nucleophile such as water attacks the sulphur atom and makes a five co-ordinate intermediate that is stabilised by the calcium ion, which collapses by a breakage of the bond between the sulphur and the glycosidic oxygen, releasing the glycosaminoglycan and leaving the sulphate bound to residue 91[159] (Scheme 2.3).



Scheme 2.3 : Proposed reaction of ester cleavage for ASB[226]

Like ASB, ASA also shares the unique post-translational modification of a conserved cysteine residue. In this case cys69, which is converted to formylglycine, where the thiomethylene group of the side chain is replaced by an aldehyde group.[226] The conserved residues surrounding the active site pocket are Asn282, Asp281, Cys69, Asp29, Asp30, Lys302, His405, Val91, Arg73 and Lys123.[227]

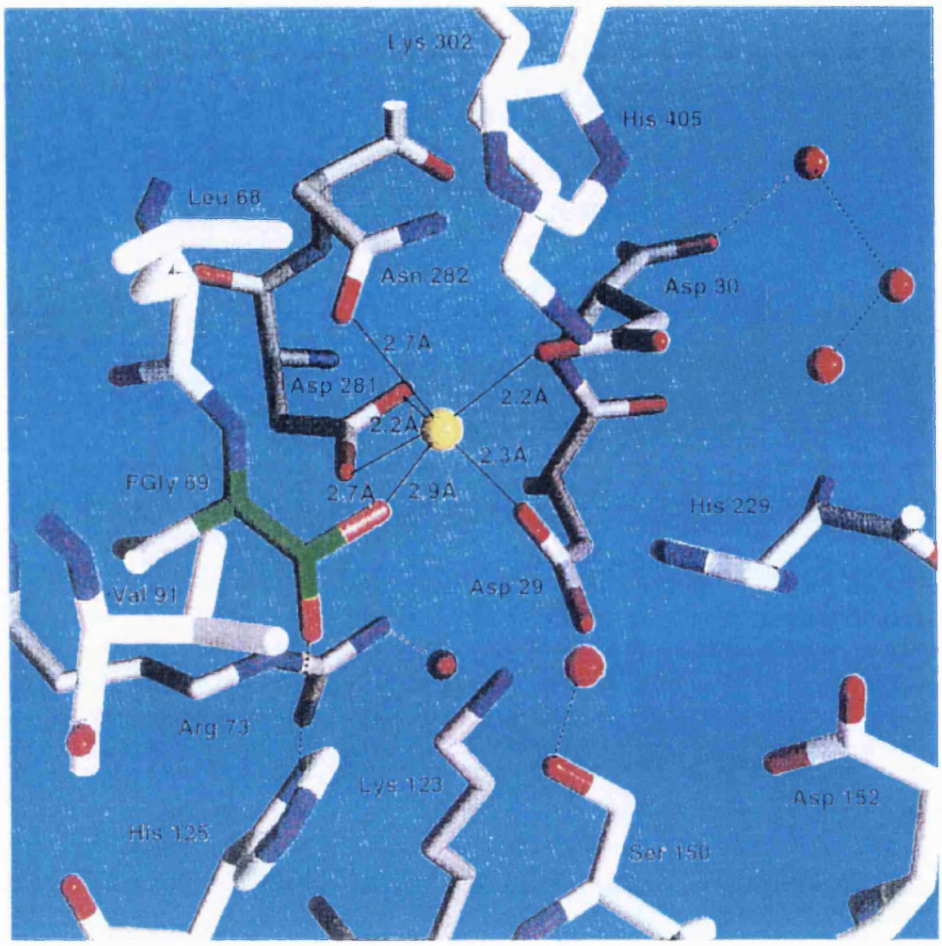


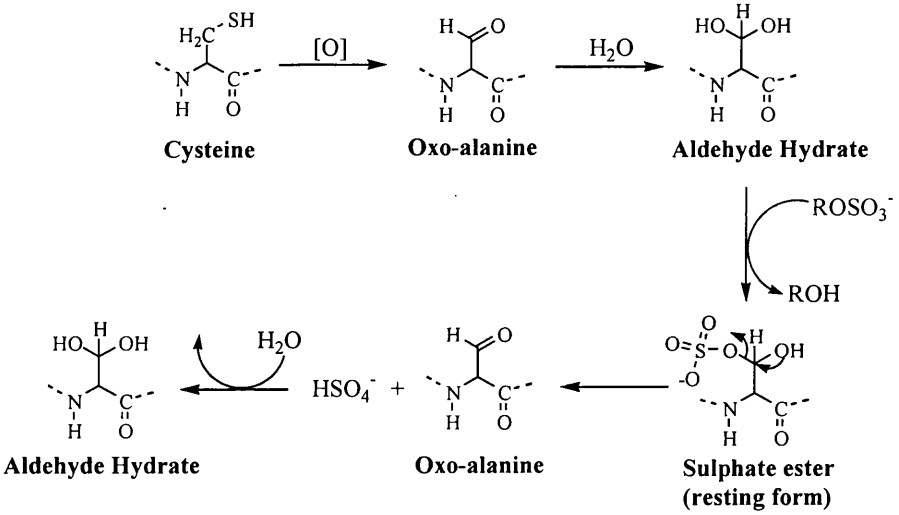
Figure 2.22 : Sketch view of the catalytic site of ASA[227]

(A) **(B)**

Figure 2.23 : Structures of the adducts formed by ASB and ASA

In ASA the formylglycine residue is located in a positively charged substrate-binding pocket in which a Mg^{2+} is co-ordinated (Figure 2.22). In contrast to ASB, which is a sulphate adduct of the aldehyde **(A)**, the electron density at the formylglycine side chain of ASA is shown to be an aldehyde hydrate **(B)**[153] (Figure 2.23).

Using the crystal structures, two different catalytic mechanisms have been proposed for ASB and ASA. The sulphate sulphur atom undergoes a nucleophilic attack by one of the geminal oxygen atoms of the aldehyde. The Mg^{2+} increases the electrophilicity of the S atom and facilitates the nucleophilic attack of the hydrate O atom leading to the formation of a sulphated enzyme intermediate, which is the same covalent intermediate as the resting form observed for ASB (c.f. Scheme 2.2). The aldehyde is regenerated by the elimination of the sulphate induced by the second non-esterified hydroxyl group of the covalent intermediate (Scheme 2.4). These mechanisms provide a good model to understand how the hydrolysis of sulphate esters may work in sulphatase enzyme by the sulphamate inhibitors.



Scheme 2.4 : Hypothetical mechanism of sulphuric acid ester hydrolysis by ASA[226]

In order to discriminate the two mechanisms Recksiek *et al* constructed mutant proteins of ASA and ASB[226] in which the active site formylglycine originated from a cysteine is replaced by a serine [-CH_2-OH] by site-directed mutagenesis. This leads to a sulphatase protein that can no longer be modified to the formylglycine-containing form. The results showed that the serine mutants of both ASA and ASB make one half-cycle and as a consequence, it forms a sulphoserine-containing derivative by cleaving the sulphate ester of the substrate.

If the catalytic cycle is initiated by the addition of the sulphate ester to the 3-oxogroup of the active site formylglycine as proposed for ASB, only the wild-type sulphatases but not the serine mutants should be able to cleave the sulphate ester and to form a sulphated intermediate. But, substitution of the formylglycine by serine abolished the reaction of sulphatases.[226]

If on the other hand the sulphate ester reacts with an active site aldehyde hydrate as proposed for ASA, the serine mutant could be able to form a covalently sulphated intermediate. But the results strongly suggests that the wild type sulphatases initiate sulphate ester cleavage by transesterification (Scheme 2.3), which requires a nucleophilic hydroxyl group at the catalytic residue, which in sulphatases can only be initiated by hydration of the formylglycine residue.[226]

2.8 Steroid sulphatase inhibition by EMATE and related synthetic sulphamates

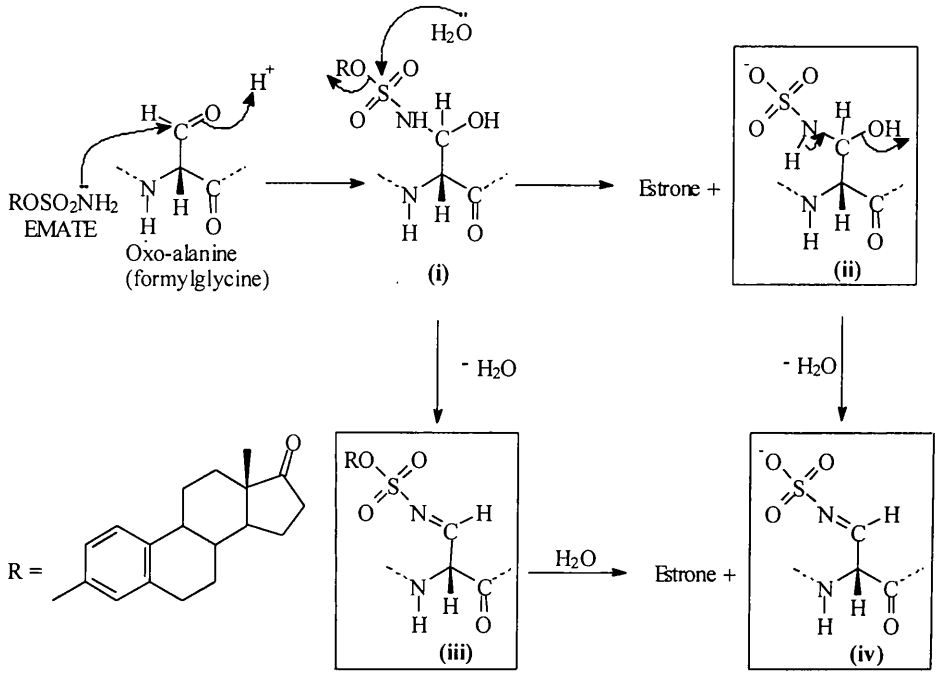
Since it has been shown that the catalytic site amino acid residues for E1-STS activity amongst sulphatase enzymes are conserved. It is anticipated that steroid sulphatase would share a similar mechanism of sulphate hydrolysis to those proposed for ASA and ASB. It is therefore reasonable to re-examine the previously proposed mechanisms for EMATE on reflection of the two recently proposed catalytic pathways for ASA and ASB. EMATE could have inactivated steroid sulphatase by one or a combination of the following proposed mechanisms.

2.8.1 Involvement of the oxo-alanine residue

The first possible mechanism is analogous to that proposed by Bond *et al*, for sulphate hydrolysis.[159] The resting sulphated derivative of oxo-alanine could be in equilibrium with its free form and EMATE could be expected to attack the electrophilic aldehyde group.

As shown in scheme 2.5, initially, a nucleophilic attack by the amino of the sulphamate group on the oxo-alanine takes place to give **i**. The next step involves the release of E1,

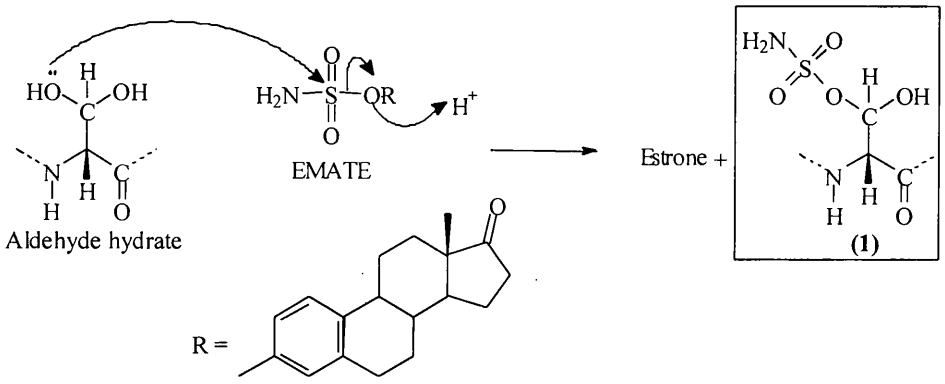
possibly as a result of displacement by a nucleophilic attack by water on the sulphur atom of the sulphamate to give the sulphamoylated residue **ii** and elimination of water takes place first to give the imino sulphamoylated form **iii,** which subsequently under goes hydrolysis by water to give **iv** with the release of E1 (Scheme 2.5).



Scheme 2.5 : Proposed mechanism for EMATE-induced inhibition of steroid sulphatases: Involvement of the oxo-alanine residue. The potential dead-end enzyme complexes are boxed. (Adapted from Woo *et al*[219])

2.8.2 Involvement of the aldehyde hydrate residue

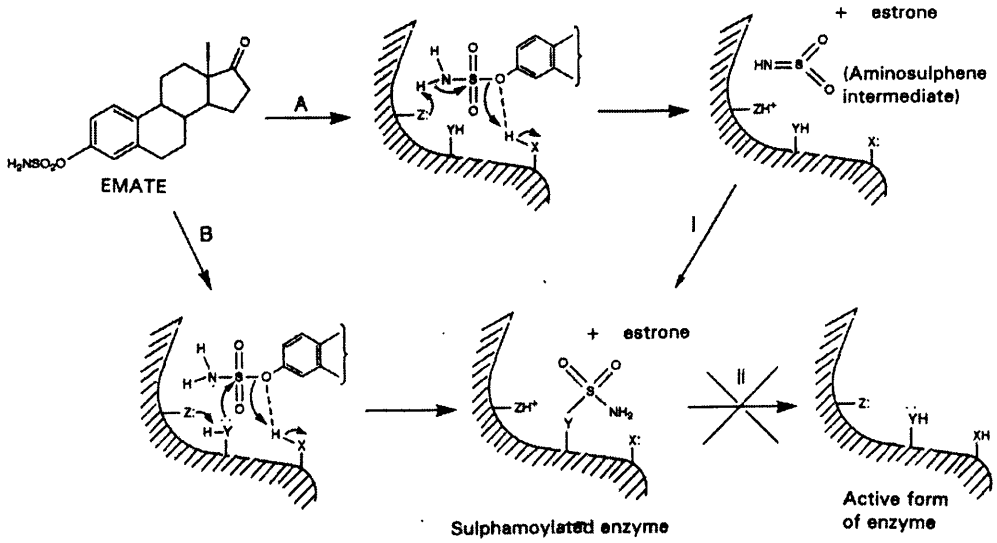
The second possible mechanism could be similar to that proposed by Recksiek *et al* for sulphate hydrolysis, i.e. a nucleophilic attack on the sulphamate group at the sulphur atom by one of the hydroxyl group of the aldehyde hydrate residue to give the sulphamoylated form **1**, which could be a dead-end product[226] (Scheme 2.6)



Scheme 2.6 : Proposed mechanism for EMATE-induced inhibition of steroid sulphatases: Involvement of the aldehyde hydrate residue. The potential dead-end enzyme complexes are boxed. (Adapted from Woo *et al*[219])

2.8.3 A non-specific or random specific sulphamoylation

When EMATE binds to E1-STS, the enzyme is inactivated when either an essential amino acid residue, which is normally sulphated during the catalysis or an important neighbouring residue, becomes irreversibly sulphamoylated. (Scheme 2.7)



Scheme 2.7 : Non-specific sulphamoylation of sulphatase by EMATE. (Adapted from Woo *et al*[219])

Two mechanisms for this type of sulphamoylation are feasible : it may either be mediated by (A) the formation of a reactive electrophilic aminosulphene intermediate, which rapidly sulphamoylates the active site, from the anion of EMATE *via* a E/cb process possibly initiated by an enzyme catalysed proton abstraction from the amine on the sulphamate group (Scheme 2.7 A) and most likely stimulated by H-bonding to the bridging O-atom; or (B) a direct nucleophilic attack by an amino acid residue at the sulphur atom of the sulphamate group (Scheme 2.7 B). Both mechanisms will rapidly lead to a sulphamoylated enzyme intermediate, which cannot be hydrolysed by water to regenerate the active form of the enzyme in the same manner or rapidly as the sulphoenzyme intermediate resulting from the hydrolysis of estrone sulphate thus rendering the enzyme irreversibly inhibited.

Analysis of pH dependence of enzyme activity shows that there are two ionisable groups with pK_a values of 7.2 and 9.8 that are involved in the enzyme inactivation by EMATE. Initial studies using rose bengal inactivated the amino acid histidine and suggested that histidine may play a role in the catalytic mechanism. Another amino acid, tyrosine was also suspected to be involved in the transfer between a tyrosine-enzyme substrate and a phenolic acceptor substrate since the pK_a of tyrosine is 9.8 and correlates with the requirement for the inactivation of EMATE. However the recent finding shows that tyrosine is not present in the conserved active site of the enzyme, therefore some other amino acids with a similar pK_a must be involved. The most likely candidate with a similar pK_a would be lysine, which has a pK_a of ~10 in proteins.[228]

2.9 Aims of this project

This project aims broadly (i) to design and synthesise a range of non-steroidal inhibitors, which would be as potent as EMATE and 667 COUMATE, and (ii) to build up a more comprehensive structure-activity relationship (SAR) for these coumarin sulphamates. Therefore I focused on synthesising coumarin sulphamate derivatives with alkyl chains and other functionalities at the C-3 and/or C-4 positions and a complete series of tricyclic coumatin sulphamates as potentially effective inhibitors of E1-STS. It is likely that other herterocyclic ring containing sulphamates would also

show some interesting activities. Therefore several novel indole sulphamate derivatives have also been explored as an alternative to the coumarin system, which inhibit E1-STS enzyme.

CHAPTER 3

CHAPTER 3

C-3 or/and C-4 substituted coumarin sulphamates

3.0 Background

Coumarins are ubiquitous secondary metabolites isolated from medicinal plants. Natural coumarin (2H-1-benzopyran-2-one) can be effectively extracted from the plant *Mikania glomerata* in large quantities.[229] Over the years, a number of coumarin-containing natural and synthetic drugs have been discovered and shown to have useful biological activities. Apart from use as a food and fragrance ingredient, derivatives of coumarins are useful in photochemotherapy, antitumoural, anti-HIV therapy, CNS-active drugs and most importantly as anticoagulants.[230] In addition, some of the compounds containing the coumarin ring system were found to possess estrogenic and anti-estrogenic activities such as coumestrol[90].

A large number of coumarin derivatives have shown to possess low toxicity and undesirable side effects, except in some cases. In 1988, synthetic coumarin was launched in France for the adjuvent therapy of lymphoedema of the upper limb following radio-surgical treatment of breast cancer and shown to have a potential hepatotoxicity, which is probably due to the production of a reactive metabolite in some patients exhibiting a coumarin 7-hydroxylation deficiency. Studies have shown that coumarin and 7-hydroxycoumarin were nearly equitoxic, whereras 4-hydroxycoumarin was nearly 2-fold less toxic than coumarin on an equimolar basis.[231]

Our focus on the coumarin ring system was enhanced with the potent activities obtained for COUMATE **(2-70)** and 3,4-dimethylcoumarin-7-*O*-sulphamate **(2-74)** (Table 2.4, Chapter 2). Therefore, to understand any relationship between the potency and hydrophobicity in two ring compounds, we have further explored the concept of coumarins and related molecules as templates to design non-steroidal

sulphatase inhibitors for the endocrine therapy of hormone dependent breast cancer by using COUMATE as the lead compound.

3.1 Synthesis

The compounds synthesised in this project belong to three different series. **(A)** Alkyl groups of increasing carbon chain lengths and other functionalities at the C-4 position of the coumarin ring and **(B)** alkyl groups of increasing carbon chain lengths and other functionalities at the C-3 position and a methyl group at the C-4 position of the coumarin ring, and **(C)** alkyl groups or/and other functionalities at the C-4 and C-3 positions of the coumarin ring. They can be represented by the general structures as shown below: (Figure 3.1)

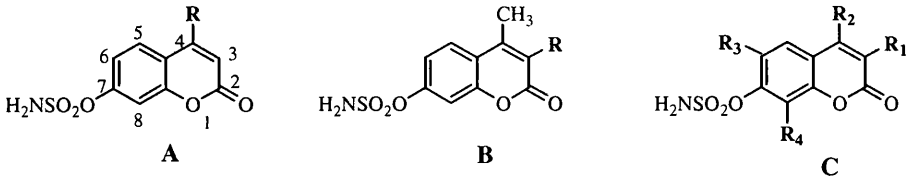
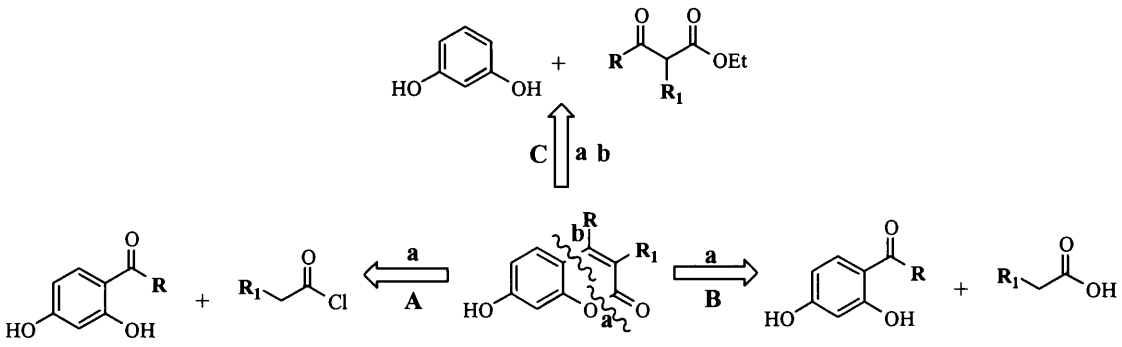


Figure 3.1 : The general structures of the compounds synthesised in this project. **A:** 4-Substituted series, **B:** 3-Substituted-4-methyl series, **C:** Other substituted series

Retrosynthetic analysis gives several possible synthetic routes to achieve these series of sulphamates as shown in Scheme 3.1. The coumarin ring can be disconnected conveniently in two ways, **a** and **b** as indicated. One highly used method is *via* route A, which involves a condensation of 2,4-dihydroxyphenyl ketones with alkylacetyl chloride. This method has been repeatedly reported to furnish the desired coumarins in high yields.[232] Another possible approach was to go *via* route B, which involves the condensation of 2,4-dihydroxyphenyl ketones with the appropriate phenylacetic acid.



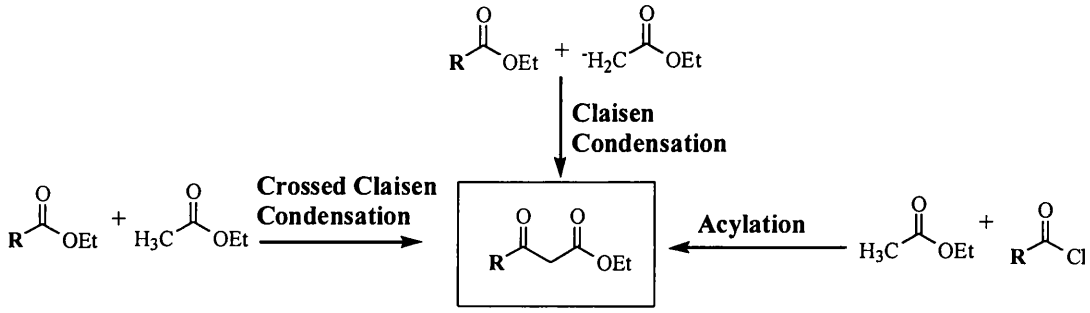
Scheme 3.1 : Retrosynthesis of hydroxy coumarins.

One other possible starting point was to synthesise the corresponding β-keto esters followed by the Pechmann synthesis of hydroxycoumarins *via* route C. For our purpose this route was primarily considered since the target coumarins can be relatively easily prepared from two similar-sized fragments and thus the forward synthesis would be convergent. The only synthetic problem would be the synthesis of various β-keto esters since most are not available commercially. The 7-hydroxycoumarins synthesised are subsequently sulphamoylated with freshly prepared sulphamoyl chloride to form the corresponding coumarin sulphamates. The Pechmann condensation reaction is now further discussed and explained in the context of how it was used.

The alkanoylacetate esters required as starting material for the coumarins in the 4-alkyl series **(A)** (Figure 3.1) of compounds are commercially unavailable and therefore, had to be synthesised. One of the more general and useful approaches involves the acylation of diethyl malonate followed by partial hydrolysis and subsequent decarboxylation of only one of the two ester groups in the acylated malonate intermediate. However, hydrolysis and decarboxylation of both ester groups may occur leading to an alkyl ketone by-product. Alternatively, both ester groups may be left unhydrolysed resulting in contamination by the acylated malonate intermediate. In some cases, retro-condensation takes place to regenerate the carboxylic acid starting material. In recent years, several more practical and

reliable procedures using stronger acids such as malonic acid,[233] Meldrum's acid (pK_a = 5)[234,235] and mixed malonate esters such as 'butyl ethyl malonate[235] have been developed, which attempt to avoid these problems (Scheme 3.2).

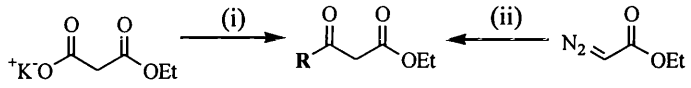
The most common ways to synthesise alkanoylacetates are by Claisen condensation reaction, which involves the condensation of an ester enolate with an ester, crossed Claisen condensation, which involves esters with no α-hydrogen and acylation of enolate anions with an acyl chloride or an ester (Scheme 3.2). Irrespective of the poor yield noted in the Claisen condensation reaction, by self-condensation of the esters, it is a widely employed synthetic procedure. The yields can be improved by using fairly strong bases such as sodium hydride, sodium amide and lithium diisopropylamide (LDA) to make the formation of the ester enolate anion essentially irreversible.



Scheme 3.2 : Ways of synthesising alkanoylacetates

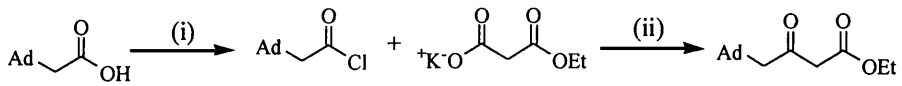
The synthetic method used in this work to synthesise the required alkanoylacetates was by treating the inexpensive ethyl potassium malonate with the corresponding acid chloride in the presence of magnesium chloride ($MgCl_2$), triethylamine (Et_3N) and acetonitrile (CH_3CN) as the solvent[236] (Scheme 3.3). This method has the advantage of being relatively safe, clean, economical, and suitable for scaling up with product produced in high yield and purity, which is free from any unnecessary side products. Rathke and Cowan have shown that the combination of anhydrous

$MgCl_2$ and Et_3N provide a strong enough base system for metallating ethyl potassium malonate.[237] They also found that the reactions failed when $MgCl_2$ was replaced with other metal chlorides such as $ZnCl_2$, $CuCl_2$, $FeCl_3$, $TiCl_4$, LiCl and $AlCl_3$.



Scheme 3.3 : Synthesis of alkanoyl acetoacetate for the preparation of 4-alkylcoumarin sulphamate. (i) (a) MeCN, $MgCl_2$, Et_3N, 10-25°C, 2.5 h (b) RCOCl, Et_3N, 0°C, 0.5 h, R.T. 12 h. (ii) RCOH, $SnCl_2$, CH_2Cl_2, R.T. 3 h.

The number of equivalents of reagents used in the reaction determines the yield of the product obtained. For aromatic acid chlorides which have electron-withdrawing substituents such as fluoro, chloro or nitro groups, 2.1 equivalents of potassium ethyl malonate, 2.5 equivalents of $MgCl_2$ and 2.2 equivalents of Et_3N are optimal and the yield is around 90%. On the other hand, aliphatic acid chlorides or aromatic acid chlorides containing electron-donating substituents generate side products, which are minimised by employing extra equivalent of Et_3N to obtain the alkanoylacetate in high yield. 1-Adamantaneacetic acid was converted to the corresponding acid chloride by reacting with thionyl chloride ($SOCl_2$); reaction with potassium ethyl malonate gave 4-adamantan-1-yl 3-oxo-butanoate **(54)** (Scheme 3.4).



Scheme 3.4 : Synthesis of 4-adamantan-1-yl 3-oxo-butanoate **(54)** (i) $SOCl_2$, DMF, reflux (ii) (a) MeCN, $MgCl_2$, Et_3N, 10-25°C, 2.5 h (b) Et_3N, 0°C, 0.5 h, R.T. 12 h.

The mechanism of the reaction is expected to be as shown in Figure 3.2. Et_3N removes the α-hydrogen of the ethyl potassium malonate and produces a carbanion, which behaves as a nucleophile attacking the acid chloride with the displacement of the chloride ion.

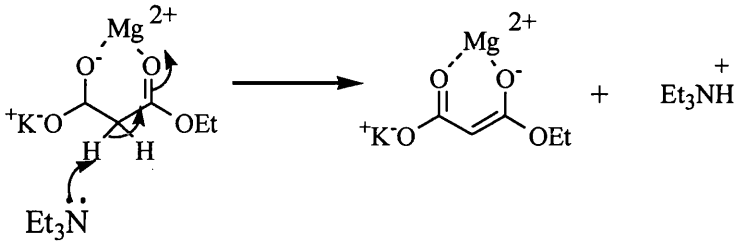


Figure 3.2 : Metallation of ethyl potassium malonate by $MgCl_2$ to form the anion.

$MgCl_2$ acts as a Lewis acid and assist the formation of the anion. The Mg^{2+} forms a complex co-ordination with the carbonyl O atoms (Figure. 3.2) and hence the abstraction of the α-hydrogen by Et_3N to generate the anion is facilitated.

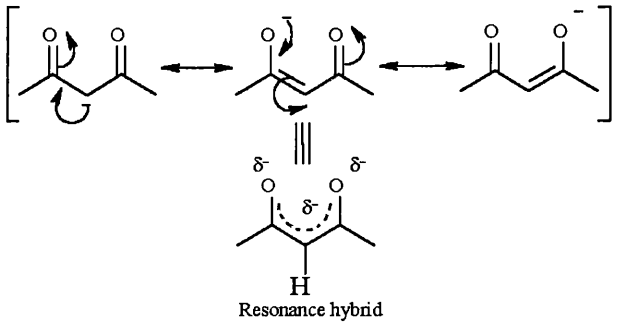
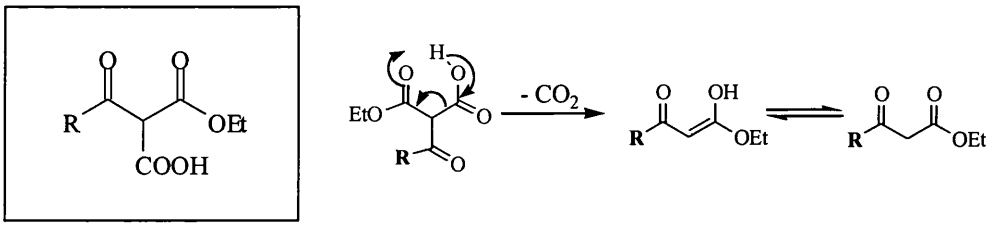


Figure 3.3 : The resonance hybrid formed by the delocalisation of electrons

The two flanking carbonyl groups of malonate markedly increase the acidity of the α-hydrogen (pK_a = 10 – 14) and the carbanion formed is highly stabilised due to delocalisation of electrons (Figure 3.3). The β-keto ester is the most acidic molecule in the sequence of this reaction because of the two carbonyl groups which stablise the α-carbanion. The equilibrium of the reactions is shifted towards the

anion again because of its stability. In practice, the neutral β-keto ester is recovered through acidification of the reaction mixture. Upon acidification of the reaction mixture, the resulting β-keto-α-carboxy ester **(I)** readily undergoes decarboxylation[238] (Figure 3.4) to yield CO_2 and the desired alkanoylacetate (Figure 3.4). When the acid decarboxylates, it goes through a six-membered transition state as shown in figure 3.4, which occurs at relatively low temperatures.



(I)

Figure 3.4 : Mechanism of decarboxylation

β-Ketoesters can also be prepared efficiently by reacting the corresponding aldehyde with ethyl diazoacetate in the presence of a catalytic amount of tin(II) chloride ($SnCl_2$) (Scheme 3.3). The two most noteworthy aspects of this method are its selectivity and the mild conditions involved. The reaction is insensitive to atmosphere and is completed between 1-2 h at R.T. This reaction can be catalysed by various Lewis acids, such as BF_3, $ZnCl_2$, $ZnBr_2$, $AlCl_3$, $SnCl_2$, $GeCl_2$ and $SnCl_4$ but the highest yield is obtained with $SnCl_2$.[239] Even though other common organic solvents can be used, CH_2Cl_2 is often employed because it gives the best results and can be easily removed.

The mechanism of the reaction is likely to have proceeded *via* a betaine intermediate, followed by a preferential migration of the aldehyde hydrogen to the β-carbon i.e. a 1,2-hydride shift producing the required β-keto ester and N_2 as products. The possible mechanism of this reaction is as follows : (Figure 3.5)

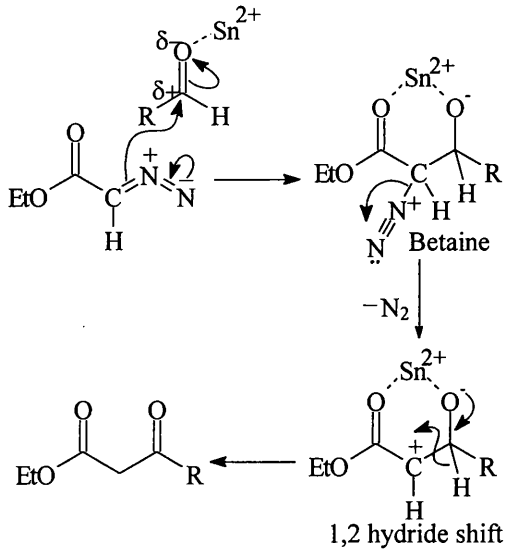
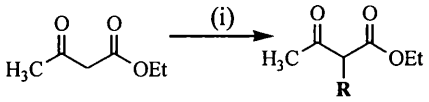


Figure 3.5 : Proposed mechanism for the synthesis of ethyl alkanoylacetates by $SnCl_2$

Several efficient and convenient methods have been described for the alkylation of ethyl acetoacetate, by alkylation of sodium acetoacetic esters in by alkyl halides by using different solvents such as DMF and different alcohols.[240,241] These reactions require long times and the use of metallic sodium. Therefore, the α-alkylacetates required for the synthesis of coumarins in the 3-substituted-4-methyl series were prepared by a convenient method by treating a solution of ethyl acetoacetate in CH_2Cl_2 with the corresponding alkyl bromide in the presence of potassium carbonate (K_2CO_3) and tetrabutylammonium chloride (Bu_4NCl)[242] (Scheme 3.5).

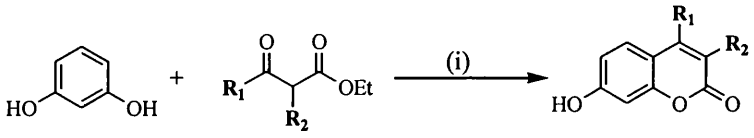


Scheme 3.5 : Synthesis of α-alkylacetoacetates for the 3-alkyl-4-methylcoumarin sulphamates. (i) RBr, K_2CO_3, H_2O, Bu_4NCl, CH_2Cl_2, reflux, 40 h.

Alkyl bromides are chosen as the alkylating agent in place of alkyl iodides, since they are readily available, relatively cheaper and the bromide ion is a better leaving

group than Cl but similar to I. The phase-transfer catalyst tetrabutylammonium iodide (Bu_4NI) is insoluble in water but soluble in all common organic solvents. Great interest has now been developed in such phase transfer processes. The principle is to generate a carbanion by K_2CO_3, which is normally insoluble in organic solvents and use the phase-transfer catalyst to transport this anion into the organic phase, then allow this anion to react in the organic phase. The reactive anion generated in this experiment was the ethyl acetoacetate carbanion at the α-carbon. Once dissolved by the phase transfer catalyst in the organic solvent in this case CH_2Cl_2, this anion reacts with the alkylating agent, which is the alkyl bromide to give the acetoacetic ester alkylated product.[243] All the compounds were easily purified by flash chromatography with $CHCl_3$.

All the parent coumarins were prepared under Pechmann conditions from the corresponding β-keto ester and resorcinol in the presence of an equimolar mixture of trifluoroacetic acid (CF_3COOH) and concentrated sulphuric acid (H_2SO_4) (Scheme 3.6) warmed from ice-water temperature to R.T.



Scheme 3.6 : The Pechmann synthesis of 4-alkyl and 3-alkyl-4-methyl substituted-7-hydroxycoumarin (i) Conc.H_2SO_4/ CF_3COOH, 3 h, 0°C to R.T.

The use of a 1 : 1 mixture of conc. H_2SO_4 and conc. CF_3COOH as the condensing agent for the Pechmann synthesis of coumarins was first described by Hua *et al*[244] and in our hands such mixture has been found to be as effective as using conc. H_2SO_4 alone, which is playing a role as a catalyst. The role of CF_3COOH in this reaction is not entirely clear, although it might be acting as an organic solvent and lowering the viscosity of the reaction mixture rendering the stirring process more efficient.

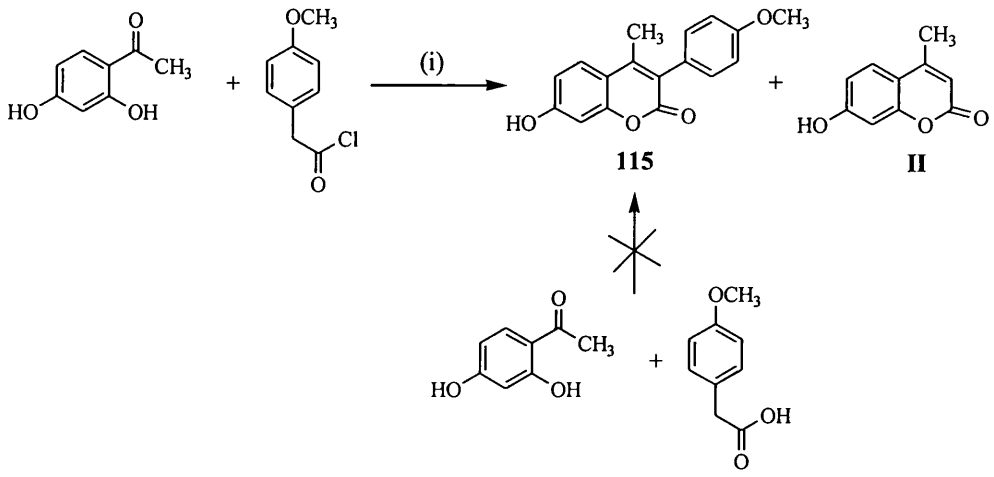
The synthesis of 7-hydroxy-3-(4-methoxyphenyl)-4-methylcoumarin **(115)** was not possible using Pechmann conditions (Scheme 3.6) since the corresponding β-keto ester cannot be prepared directly in the same manner described above (Scheme 3.4). In the literature several methods have been reported for the preparation of 3-methyl-4-phenyl derivatives of coumarin.[245,246,247,248] Several similar 3-substituted coumarins have been reported to be prepared by various other methods. 2-Hydroxyacetophenone with phenylacetyl chloride in acetone using anhydrous potassium carbonate as the base also appears to produce the required coumarins in high yields.[232,249] This method is only viable for phenyl-containing compounds and found to be unsuccessful with alkyl coumarins.[232] Direct condensation of 2-hydroxybenzaldehydes with phenylacetic acid by the Perkin method does not give the products in good yields.[250]

Therefore, an attempt was made to synthesise the desired 7-hydroxy-3-(4-methoxyphenyl)-4-methylcoumarin **(115)** by Hans *et al* using 2,4-dihydroxyacetophenone with phenylacetic acid in the presence of dicyclohexylcarbodiimide (DCC) in dimethylsulphoxide (DMSO)[251] (Scheme 3.7). Complex products were resulted when heated the reaction mixture at 100-110°C for 24-28 h, which could not be separated into individual components and also the reaction did not take place when attempted at room temperature or at lower temperatures.

Alternatively, **115** was then synthesised using the general method described by Sabitha *et al* by condensation of 2,4-dihydroxyacetophenone with 4-methoxyphenylacetyl chloride using aqueous potassium carbonate (K_2CO_3) (20%) as base and CH_2Cl_2 as the solvent in the presence of a phase-transfer catalyst, tetrabutylammonium sulphate ($Bu_4N.HSO_4$), which furnished the desired hydroxy coumarin in good yield[252] (Scheme 3.7).

2,4-Dihydroxyacetophenone was insoluble in CH_2Cl_2 therefore it was first

dissolved in a small amount of THF and then diluted with CH_2Cl_2. Aqueous K_2CO_3 was used as a base instead of potassium hydroxide because the stronger base ring opened the coumarin resulting in the formation of the potassium salt of a substituted 2-hydroxycinnamic acid derivative, which remains in the aqueous layer during the work-up. Although acidification of the aqueous layer regenerates the desired coumarin, the use of aqueous K_2CO_3 obviates this complication.



Scheme 3.7 : Synthesis of 7-hydroxy-3-(4-methoxyphenyl)-4-methylcoumarin **(115)** (i) K_2CO_3, CH_2Cl_2, $Bu_4N.HSO_4$, 3-4 h, R.T.

Two pale yellow compounds were isolated from the brown crude suspension upon flash chromatography. When visualised under the UV light, one of them was found to be fluorescent and the other was UV active but not fluorescent. The NMR spectral analysis indicated that the fluorescent compound isolated was without the 3-methoxyphenyl group **(II)** (Scheme 3.7) and the non-fluorescent compound was the desired product, which was supported by the mass spectral and combustion analyses.

The sulphamoyloxy group has been identified as an active pharmacophore for the inhibition of E1-STS. Several different classes of sulphamoylated drugs have been synthesised and have shown to be active inhibitors. In the earlier work, crude

crystalline sulphamoyl chloride prepared according to the method described by Appel and Burger was used directly on phenolic compounds.[253] Sulphamoyl chloride was prepared by treatment of chlorosulphonyl isocyanate with freshly distilled formic acid (Scheme 3.8).

$$ClSO_2N{=}C{=}O \quad + \quad HCOOH \xrightarrow{\text{(i)}} ClSO_2NH_2 \quad + \quad CO_2 \quad + \quad CO$$

Scheme 3.8 : Preparation of sulphamoyl chloride (i) Anhy. toluene, N_2, 12 h, 0°C to R.T.

However, sulphamoyl chloride is very hygroscopic and decomposes readily even on storage at low temperature, which makes the continuous handling of this compound very difficult. This problem was overcome by storing freshly prepared crude sulphamoyl chloride as a solution in freshly distilled anhydrous and sulphur-free toluene under nitrogen at low temperature.[194] During the reaction carbon dioxide and carbon monoxide were formed. The mechanism for the synthesis of sulphamoyl chloride reaction can be proposed as depicted in figure 3.6.

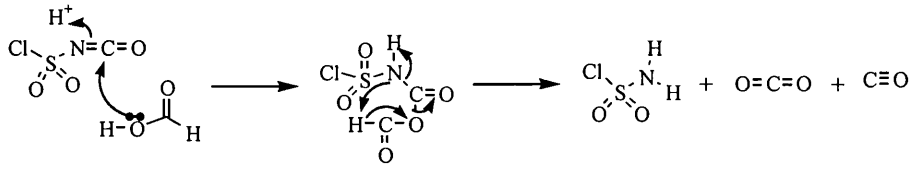
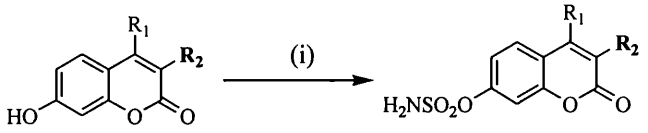


Figure 3.6 : Proposed mechanism for the synthesis of sulphamoyl chloride from chlorosulphonyl isocyanate and formic acid.

No titration was attempted on this sulphamoyl chloride solution whose molarity was estimated according to the weight of the original crude sulphamoyl chloride obtained after workup. An appropriate volume of solution was freshly concentrated *in vacuo* immediately before use.

The sulphamoylation reaction was performed by treating the hydroxycoumarins with an excess of (~ 5 equiv.) sulphamoyl chloride after treating with one equivalent of NaH as described previously by Woo *et al*[214] (Scheme 3.9).



Scheme 3.9 : The sulphamoylation reaction of 7-hydroxylated coumarins (i) Anhydrous DMF, NaH, N_2, 0°C and H_2NSO_2Cl, 0°C to R.T.

The NaH deprotonates the 7-hydroxy group of the coumarin to form the corresponding sodium phenolate. This reaction is accompanied by a colour change from a colourless mixture to a dark yellow suspension, due to the delocalisation of electrons in the coumarin ring. Upon addition of sulphamoyl chloride, the yellow colour disappears and a milky suspension results. The phenolate reacts with the sulphamoyl chloride to give the desired coumarin sulphamate and NaCl. The solvent DMF and NaCl were removed during the work-up and the organic products were extracted into ethyl acetate.

An AA'BB' relationship was observed for the four phenyl protons of 3-methoxyphenyl group in the NMR spectra for the compounds **115** and 3-(4-methoxyphenyl)-4-methylcoumarin-7-*O*-sulphamate **(116)** (Figure 3.7). Two sets of doublets corresponding to two protons each were observed at δ 6.93 ppm and δ 7.31 ppm with coupling constants of 8.6 Hz. This is characteristic of the aromatic proton resonance for a *para* substituted benzene ring. The two pairs of protons, which are magnetically not equivalent are coupled together to appear as a doublet, but actually has a number of extra lines, of which the inside lines are the most noticeable.

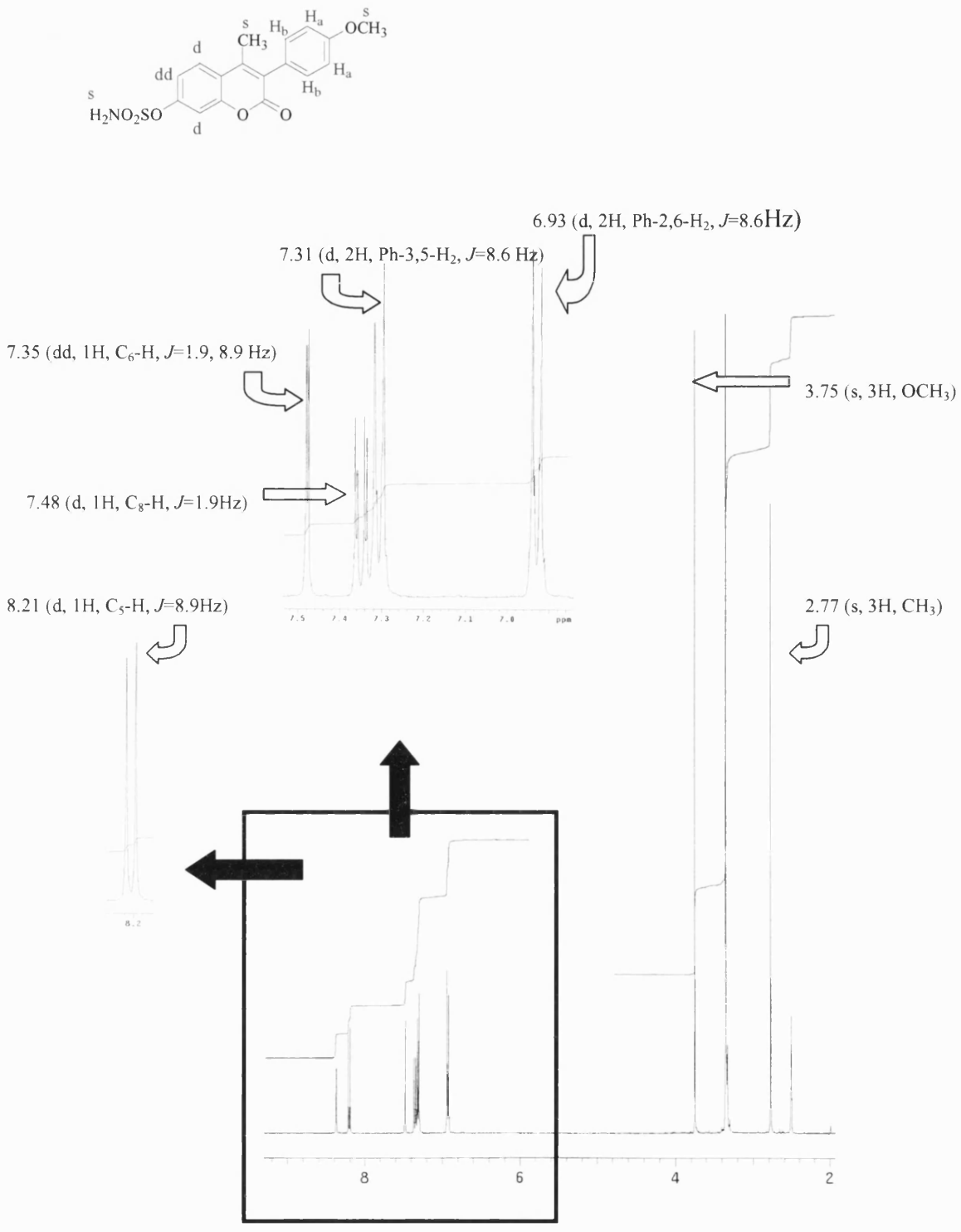
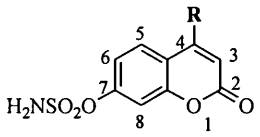


Figure 3.7 : NMR of 3-(4-methoxyphenyl)-4-methylcoumarin-7-*O*-sulphamate **(116)**.

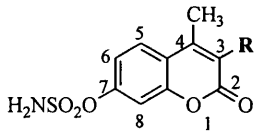
For proton NMR spectral analysis, the compounds were dissolved in deuterated solvents such as chloroform and DMSO-d_6. The keto esters and some 7-hydroxycoumarins are soluble in $CDCl_3$. The poorly soluble compounds and the sulphamates were dissolved in DMSO-d_6. All the compounds were fully characterised by spectroscopic and combustion analysis. The yields of the sulphamates were generally poor therefore, improved significantly by using excess of sulphamoyl chloride (≥ 5 equiv.). 40-50% of the unreacted phenolic starting material was isolated.

The coumarin sulphamates prepared for this work are summarised as follows:



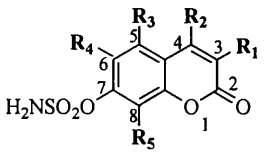
Compound	R	Compound	R
3	$CH_3(CH_2)_3$	**32**	^{i}Pr
6	$CH_3(CH_2)_4$	**34**	^{t}Bu
9	$CH_3(CH_2)_5$	**36**	CH_2OCH_3
12	$CH_3(CH_2)_6$	**38**	CH_2Cl
15	$CH_3(CH_2)_7$	**40**	C_6H_5
18	$CH_3(CH_2)_8$	**43**	CH_2Ph
21	$CH_3(CH_2)_9$	**46**	CH_2CH_2Ph
24	$CH_3(CH_2)_{10}$	**49**	$PhCH_2CH_3$
27	$CH_3(CH_2)_{11}$	**52**	C_6H_{11}
30	$CH_3(CH_2)_{12}$	**56**	CH_2-adamantyl

Table 3.1 : Structures of 4-substituted coumarin-7-*O*-sulphamates



Compound	R	Compound	R
59	$CH_3(CH_2)_4$	**89**	$CH_3(CH_2)_{14}$
62	$CH_3(CH_2)_5$	**91**	Cl
65	$CH_3(CH_2)_6$	**93**	Ph
68	$CH_3(CH_2)_7$	**95**	CH_2Ph
71	$CH_3(CH_2)_8$	**98**	CH_2CH_2Ph
74	$CH_3(CH_2)_9$	**101**	$(CH_2)_3Ph$
77	$CH_3(CH_2)_{10}$	**104**	$CH_2C_6H_{11}$
80	$CH_3(CH_2)_{11}$	**107**	$(CH_2)_2C_6H_{11}$
83	$CH_3(CH_2)_{12}$	**116**	Ph-OCH_3
86	$CH_3(CH_2)_{13}$		

Table 3.2 : Structures of 3-substituted-4-methylcoumarin-7-*O*-sulphamates



	R_1	R_2	R_3	R_4	R_5
110	$CH_3(CH_2)_8$	$CH_3(CH_2)_8$	H	H	H
112	$Ph(CH_2)_3$	CH_3	H	CH_3CH_2	H
114	$Ph(CH_2)_3$	CH_3	H	H	CH_3

Table 3.3 : Structures of other substituted coumarin-7-*O*-sulphamates

3.2 Results

Biological testing of the compounds are carried out by Dr. A. Purohit and Ms. B. Malini at the Department of Endocrinology and Metabolic Medicine, St. Mary's Hospital Medical School, Imperial College, London.

The biological evaluation of the novel synthetic entities has shown that all the compounds synthesised in the coumarin series with alkyl groups of increasing carbon chain lengths and other substituents at the C-3 and/or C4 positions of the coumarin rings are more potent E1-STS inhibitors than EMATE **(2-20)** and COUMATE **(2-70)**. All the compounds were tested in MCF-7 cells and placental microsomes at various concentrations. (Tables 3.4, 3.5, 3.6, 3.7, 3.8 and 3.9) The screening results of some of the inhibitors at lower concentrations such as 0.01 μM and IC_{50} values are not yet available.

The available biological activity results can be summarised as follows: In the C-3 alkyl series, all the compounds tested in intact MCF-7 breast cancer cells at concentrations of 10 μM, 1μM and 0.1 μM showed inhibitory activities >96% (Table 3.6). In placental microsomes, 4-methyl-3-pentylcoumarin-7-*O*-sulphamate **(59)**, 3-hexyl-4-methylcoumarin-7-*O*-sulphamate **(62)** and 3-nonyl-4-methylcoumarin-7-*O*-sulphamate **(71)** showed inhibitory activities of 94%, 88% and 85% respectively, at 0.1 μM concentration. Compound **59** has an IC_{50} value of 12 nM in placental microsomes and showed 41% inhibition at 0.01 μM, whereas compound **62** was found to have an IC_{50} of 0.68 nM in MCF-7 cells (c.f. EMATE = 25 nM) (Table 3.7).

% Inhibition on intact MCF-7 breast cancer cells

	10 μM	1 μM	0.1 μM	0.01 μM	IC_{50} nM
3	93	91	85	*	*
6	95	92	88	52	10
9	93	90	87	*	*
12	99	95	93	*	*
15	99	97	90	*	*
18	100	98	96	90	*
21	100	98	95	86	*
24	*	*	*	*	*
27	98	95	67	22	4.3
30	*	*	96	72	3.2
32	*	*	*	*	*
34	*	*	*	*	*
36	83	52	17	*	*
38	92	82	33	6	220
40	87	79	36	*	*
43	84	70	55	8	75
46	98	95	84	39	18
49	84	73	24	6	350
52	93	80	72	37	23.5
56	*	*	*	*	*

Table 3.4 : Inhibition of the E1-STS activity in intact MCF-7 breast cancer cells by 4-alkylcoumarin sulphamates at various concentrations. * = Results not available

% Inhibition in placental microsomes

	10 μM	1 μM	0.1 μM	0.01 μM	IC_{50} *n*M
3	98	91	49	10	102
6	99	97	70	22	40
9	99	97	70	6	52
12	99	97	64	8	90
15	99	97	74	28	68
18	99	97	79	18	60
21	99	96	74	17	60
24	97	92	56	8	73
27	96	89	68	17	45
30	91	80	55	7	85
32	94	90	86	*	*
34	*	97	19	*	*
36	83	52	17	*	1000
38	94	59	19	10	600
40	*	*	*	*	200
43	99	96	61	2	64
46	98	93	53	8	82
49	85	27	*	*	2600
52	99	94	69	9	42
56	*	96	8	*	*

Table 3.5 : Inhibition of the E1-STS activity in placental microsomes by 4-alkylcoumarin sulphamates at various concentrations. * = Results not yet available

% Inhibition on intact MCF-7 breast cancer cells

	10 μM	1 μM	0.1 μM	0.01 μM	IC_{50} nM
59	100	99	97	*	*
62	100	100	99	*	0.68
65	100	100	99	*	*
68	100	100	99	*	*
71	99	99	96	57	<10
74	97	99	97	80	<10
77	*	*	*	*	*
80	*	*	*	*	*
83	*	*	*	*	*
86	*	*	*	*	*
89	*	*	*	*	*
91	87	67	29	*	600
93	*	*	75	32	25
95	97	94	91	85	1
98	*	99	98	93	1.1
101	100	99	87	*	*
104	*	*	*	*	*
107	*	*	*	*	*
116	*	*	*	*	*

Table 3.6 : Inhibition of the E1-STS activity in intact MCF-7 breast cancer cells by 3-alkyl-4-methylcoumarin sulphamates at various concentrations. * = Results not yet available.

% Inhibition in placental microsomes

	10 μM	1 μM	0.1 μM	0.01 μM	IC_{50} *n*M
59	100	99	94	41	12
62	99	99	88	13	32
65	99	97	65	23	*
68	99	98	71	21	*
71	98	97	85	21	320
74	94	79	33	3	250
77	*	*	*	*	2000
80	*	*	*	*	*
83	*	*	*	*	*
86	*	*	*	*	10000
89	*	*	*	*	*
91	*	*	*	*	*
93	99	95	65	8	54
95	*	99	94	53	8
98	100	99	91	45	33
101	*	99	47	*	*
104	*	99	74	*	*
107	*	99	37	*	*
116	*	*	*	*	*

Table 3.7 : Inhibition of the E1-STS activity in placental microsomes by 3-alkyl-4-methylcoumarin sulphamates at various concentrations. * = Results not yet available.

% Inhibition on intact MCF-7 breast cancer cells

	10 μM	1 μM	0.1 μM	0.01 μM	IC_{50} *n*M
110	*	*	*	*	*
112	99	93	36	*	*
114	*	*	*	*	*

Table 3.8 : Inhibition of the E1-STS activity in intact MCF-7 breast cancer cells. * = Results not yet available.

% Inhibition in placental microsomes

	10 μM	1 μM	0.1 μM	0.01 μM	IC_{50} μM
110	*	*	*	*	*
112	*	*	*	*	*
114	*	13	6	*	>10

Table 3.9 : Inhibition of the E1-STS activity in placental microsomes * = Results not yet available.

All the compounds in the 4-alkyl series (Table 3.4) inhibited E1-STS activity in intact MCF-7 breast cancer cells, where the inhibition ranges from 67% to 96% and in placental microsomes from 49% to 79% at 0.1 μM (Table 3.5). 4-Nonylcoumarin-7-*O*-sulphamate **(18)** and 4-decylcoumarin-7-*O*-sulphamate **(21)** are found to be the most active compounds with inhibitory activities of 90% and 86% respectively, in MCF-7 cells at 0.01 μM concentration (Table 3.4). Compounds 4-tridecylcoumarin-7-*O*-sulphamate **(30)** and 4-dodecylcoumarin-7-*O*-sulphamate **(27)** were found to have IC_{50} values of 3.2 nM and 4.3 nM, respectively in MCF-7 cells. Compound **18** showed the highest inhibitory activity of 79% at 0.1 μM concentration whereas 4-octylcoumarin-7-*O*-sulphamate **(15)** exhibited potency

at 0.01 μM concentration with an inhibition of 28% in placental microsomes preparation.

3-Benzyl-4-methylcoumarin-7-*O*-sulphamate **(95)**, 4-methyl-3-(2-phenylethyl) coumarin-7-*O*-sulphamate **(98)** and 4-isopropylcoumarin-7-*O*-sulphamate **(32)** were found to be the most potent in the series where other substituents at C-3 and/or C-4 positions of the coumarin ring with activities of 96%, 91% and 86% respectively in placental microsomes at 0.1 μM concentration (Tables 3.7 and 3.5). Compounds **95** and **98** were found to have IC_{50} values of 1 nM and 1.1 nM, respectively in MCF-7 cells. Even though, all the compounds shown to be active it was found that in general the C-3 substituted sulphamates are more potent than the C-4 substituted compounds in particular, compound **95** was the most potent inhibitor of all with an IC_{50} value of 8 nM in placental microsomes.

The parent phenolic compounds were also evaluated side by side on the same assay system and found to be inactive for inhibition of E1-STS, indicating that the sulphamoyl group is the most important motif to engender sulphatase inhibitory activity.

3.3 Discussion

Following COUMATE **(2-70)**, its derivatives, 4-ethyl **(2-75)** and 4-propyl-7-*O*-sulphamates **(2-76)** inhibited the E1-STS enzyme by 77% and 81% in placental microsomes and in MCF-7 cells by 32% and 42% μM respectively. Also, 3-ethyl **(2-77)**, 3-propyl **(2-78)** and 3-butyl-7-*O*-sulphamates **(2-79)** inhibited the enzyme by 57%, 83% and 90% in placental microsomes, and in MCF-7 cells by 92%, 95% and 97% at 0.1 μM respectively[219] (Table 2.4, Chapter 2). This increase in inhibition with the increase in alkyl chain length was encouraging and prompted the approach of synthesising further analogues of coumarin sulphamates with alkyl chains of various lengths at the C-3 and/or C-4 positions of the coumarin ring, which may lead to the development of potential sulphatase inhibitors. Therefore, a

number of various different coumarin sulphamates have been synthesised and tested for their ability to inhibit E1-STS enzyme. The majority of the compounds in these series are more potent than EMATE and COUMATE.

Although time- and concentration-dependence studies have not yet been carried out to confirm the nature of inhibition of these compounds, we anticipate from our understanding of the mechanism of action for EMATE and related compounds, that the best compounds in the coumarin sulphamate series (Figure 3.8) are also acting in a similar manner to EMATE as active site-directed inhibitors.

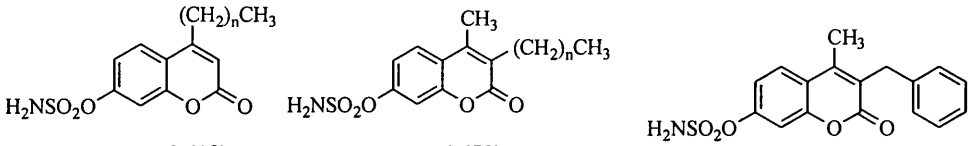
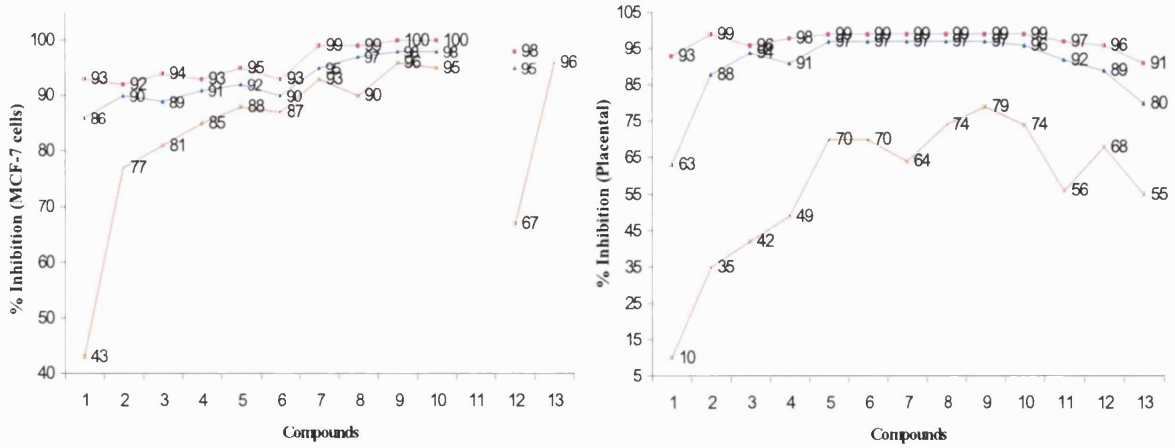


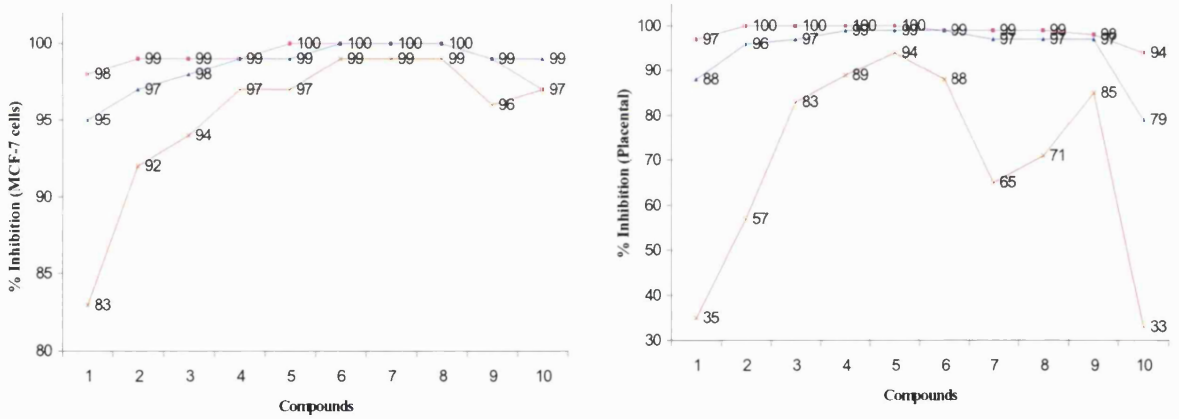
Figure 3.8 : Some potent inhibitors in the coumarin series of compounds

The consequence of having a long hydrophobic alkyl chain on the inhibition of E1-STS was shown by the *N*-alkanoyl tyramines synthesised by Li *et al*, which showed an increase in activity as the length of the alkyl chain was increased.[254,255] This observation by Li *et al* was further justified by the biological activities observed with the long alkyl chain containing coumarin sulphamates. On comparison of the results from both series have suggested that substitution with an unbranched alkyl group of the same chain length at the 3-position of the coumarin ring is more productive than that at the C-4 position as shown by the relatively higher potencies of the inhibitors in the former series (e.g. ethyl **2-75** (35%) *vs* **2-77** (57%) (Tables 2.4); nonyl **18** (79%) *vs* **93** (85%) at 0.1 μM in placental microsomes (Tables 3.5 and 3.7).



A B

Figure 3.9 : The trend of inhibition by 4-alkyl series of compounds in MCF-7 cells **(A)** and placental microsomes **(B)** at 10 μM, 1 μM, 0.1 μM concentrations.

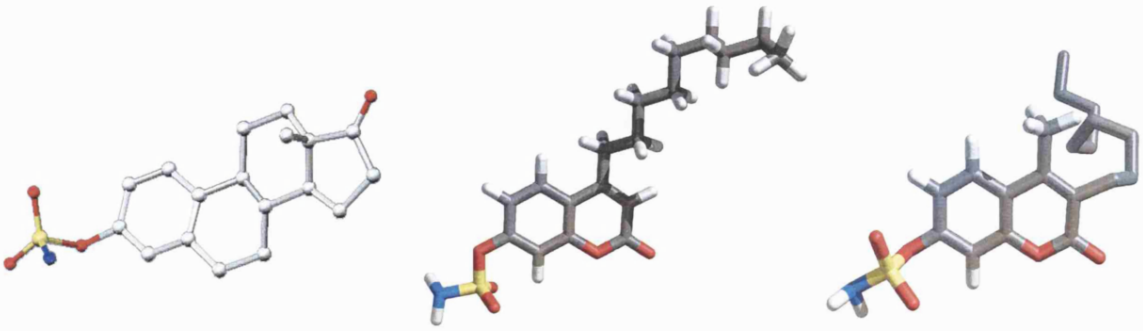


C D

The trend of inhibition by 3-alkyl-4-methyl series of compounds in MCF-7 cells **(C)** and placental microsomes **(D)** at 10 μM, 1 μM, 0.1 μM concentrations. X- axis shows the number of carbon atoms in the alkyl chain and the numbers on Y-axis indicate the % inhibition of the corresponding compound at a particular concentration.

Even though the 4-alkyl series of compounds is comparatively less potent than their corresponding 3-alkyl-4-methyl series of compounds for the same alkyl chain length a steady increase in activity was observed in both series of compounds (Figure 3.9). The % inhibition of these compounds is very high at 10 μM and 1 μM concentrations. The difference in % inhibition is clearly visible at 0.1 μM concentration and mainly in placental microsomes preparation (Figure 3.9 B and D).

It is apparent that the hydrophobic interactions between the amino acids in the enzyme active site, which naturally recognise the steroid scaffold, and the alkyl substituents are more effective when these substituents are placed at the C-3 position of the coumarin ring. Since, we have proposed that coumarin sulphamates are steroid sulphatase inhibitors by virtue of their structural mimicry of the A/B ring of EMATE, it is conceivable that the positioning of alkyl substituents with a high rotational degrees of freedom at the C-4 position of the coumarin ring may be counterproductive. The active site of steroid sulphatase, like many other enzymes with steroids as substrate, is expected to have limited accommodation for substituents at the C-1/C-11/C-12 edge of the steroid scaffold. The coumarin ring system presumably mimics the steroid rings of EMATE, and the long alkyl chains might be possessing similar hydrophobicity of EMATE, which is required for extra binding to the enzyme and thus exhibit potent STS inhibitory activities. The long chains also may be generating the opitmal spacing and hence showing certain similarity to that of the EMATE molecule. The structural comparison carried out for EMATE and the active compounds in the C-3 and C-4 alkyl series by computer modelling show the minimum energy conformation of these compounds (Figure 3.10). The flexible side chains in **18** and **71** enable these compounds to fold in such a way to create a steroid like structure, which is presumably highly preferred by the amino acid residues at the enzyme active site.

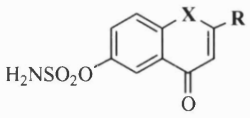


EMATE **18** **71**

Figure 3.10 : Molecular modelling of the X-ray crystal structure of EMATE on Sybyl® and pictorial representation of some of the potent inhibitors in the alkyl series compounds, 4-nonylcoumarin-7-*O*-sulphamate **(18)** and 3-nonyl-4-methylcoumarin-7-*O*-sulphamate **(71)**.

Compounds with other functionalities at the C-3 and/or C-4 position of the coumarin ring showed varying degrees of potency. Compounds **32** to **56** in the 4-substituted series (Table 3.4) showed good inhibitory activities at 10 μM, which range from 83% to 94% in MCF-7 cells and 83% to 98% in placental microsomes. (Table 3.5) At 1 μM concentration, they still showed a good inhibition, ranging from 52% to 90% in MCF-7 cells and 52% to 96% in placental microsomes. Testing at lower concentration such as 0.1 μM distinguished 4-isopropylcoumarin-7-*O*-sulphamate **(32)** to be the best compound, which showed an inhibition of 86% in placental microsomes. Interestingly, the activity drops tremendously for 4-^{t}butylcoumarin-7-*O*-sulphamate **(35)** 19% at 0.1 μM concentration, even though **35** was more active than **32** at 1 μM. This indicates that a sterically hindering bulky group is preferred at the C-4 position for the compound to fit perfectly at the enzyme active site and an isopropyl group seems to be possessing the optimal size. A similar finding was also shown by Billich *et al*[256] (Figure 3.11). Presence of bulky *tert*-butyl and adamantyl groups have shown to produce potent STS inhibitors, which was demonstrated by the 2-^{t}butyl or (1-adamantyl)-4H-chromen-

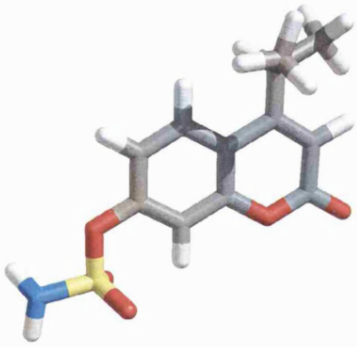
4-one-6-*O*-sulphamate and 2-(1-adamantyl)-4H-thiochromen-4-one-6-*O*-sulphamate synthesised by Billich *et al.*[256]



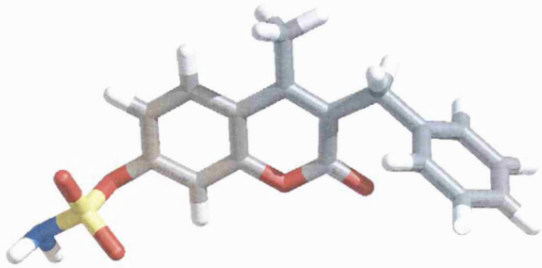
R	X	Relative IC_{50}
^{tert}Bu	O	0.4
Adamantyl	O	0.1
Adamantyl	S	0.0064

Figure 3.11 : Sulphamates reported by Billich *et al.*

Similarly, compounds **91** to **116** in the 3-substituted series also showed almost complete inhibition of > 99% at 1 μM, in placental microsomes (Table 3.6). At lower concentrations such as 0.1 μM 3-benzyl-4-methyl-7-*O*-sulphamate **(95)** was found to be the most potent inhibitor with an IC_{50} of 8 nM in placental microsomes, which is more active than EMATE and equipotent to 667 COUMATE, which is currently a phase 1 clinical trial candidate. At the enzyme active site the compound **95** is presumably folding in such a way that it mimics the steroid scaffold and the flat benzene ring provides the necessary binding to the hydrophobic amino acid residues (Figure 3.12).



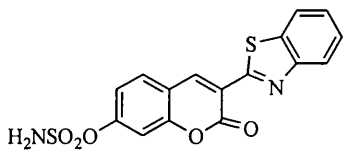
32



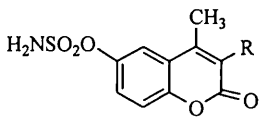
95

Figure 3.12 : A computer modelling pictorial representation of the other best inhibitors 4-isopropylcoumarin-7-*O*-sulphamate **(32)** and 3-benzyl-4-methyl-7-*O*-sulphamate **(95)**

The optimum size of the substituent is an important factor in the inhibition of E1-STS enzyme as well as the position of the substituent, spacing of atoms, and the presence and position of the sulphamoyl group on the molecule. Koller *et al* synthesised several coumarin-based compounds with pK_a values ≤ 7 and suggested that the corresponding sulphates are substrates for aryl sulphatases. The best coumarin sulphate in their series of compounds was sulphamoylated **(3-1)** by Bilban *et al* who found that it is 250 times less potent than EMATE[257] (Figure 3.11). The most likely reason for this finding is that Koller *et al* tested their compounds on arylsulphatase A and B, which show only about 30% homology to STS and their natural substrates. Galactose sulphates in sulpholipids and glucosaminoglycans are structurally different from the natural substrate of steroid sulphatase, estrone sulphate. Also, the benzothiazolyl substituent in **3-1** may be sterically too demanding for tight binding to the enzyme active site. Therefore, the substituent at the C-3 position should be something that is hydrophobic, sterically not too hindering and rigid. The 3-benzyl substituent in compound **95** seems to be possessing the necessary hydrophobicity, ideal length and also sterically acceptable for the C-3 position for a tight binding at the enzyme active site. Since the IC_{50} values were rather large for 3-phenyl-4-methyl-7-*O*-sulphamate **(93)** (54 nM) and 3-phenethyl-4-methyl-7-*O*-sulphamate **(98)** (33 nM), which contains an extra CH_2 spacer group (Table 3.7). *In vivo* studies on compound **95** are currently in progress due to the highly promising IC_{50} value of 8 nM.



3-1



3-2

Figure 3.11 : Compounds reported by Bilban *et al*

Bilban *et al* synthesised 3-phenyl derivatives with sulphamoyl groups at 7 and 6 position of the coumarin ring and found that the compounds with sulphamoyloxy

group at the 6 position **(3-2)** (Figure 3.11) is less potent than the corresponding 7-*O*-sulphamate.[257] This proves that the bridging O atom at the 7 position of the coumarin core is a prerequisite for potency.[196]

Compounds **110, 111** and **112** were synthesised in order to investigate the effects of having substituents at different positions of the coumarin ring in the enzyme activity (Tables 3.8 and 3.9). Since the nine-carbon containing compounds **15** and **71** showed good biological activities, it was interesting to see the activity of the compound, which contains a combination of both drugs. Therefore, 3,4-dinonylcoumarin-7-*O*-sulphamate **(110)** was synthesised with two long nine carbon hydrophobic chains at the C-3 and C-4 positions of the coumarin ring. Such a feature presumably helps to increase the lipophilicity of the compound to the optimum value, and to improve the binding ability at the enzyme active site. Activity results of these compounds are not yet available to understand the binding mechanism and to make a clear comparison with the other coumarin analogues.

Overall, this study has revealed that coumarin sulphamates are potential STS inhibitors, which can potently inhibit E1-STS activity in MCF-7 cells as well as in placental microsomes. However, biological activity results of all the compounds at even lower concentrations such as 0.001 μM and their corresponding IC_{50} values are required in order to get a clearer picture of the nature of the inhibition of these compounds. Identification of the most potent compound **95** produced a new lead and it may be a potential candidate for clinical evaluation.

3.4 Conclusion

We have further established in this work that coumarin sulphamates represent a key lead template for the optimaisation of potential steroid sulphatase inhibitors and that highly potent derivatives could be designed by substituting at the C-3 and/or C-4 positions of the coumarin ring with hydrophobic moieties. Diagrammatically, we can compare the steroid backbone of EMATE and the most active coumarin

sulphamates synthesised in this project (Figure 3.12).

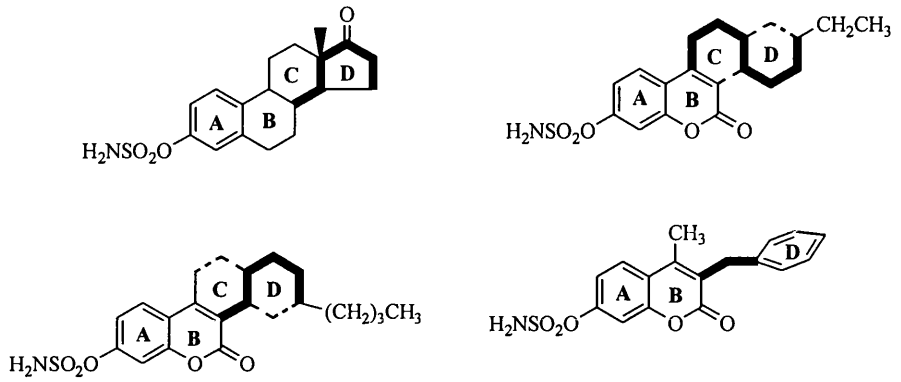


Figure 3.12 : Diagrammatic comparison of the best inhibitors, 4-nonylcoumarin-7-*O*-sulphamate **(18)**, 3-nonyl-4-methylcoumarin-7-*O*-sulphamate **(71)** and 3-benzyl-4-methylcoumarin-7-*O*-sulphamate **(95)** with EMATE.

It can be visualised that the coumarin ring mimics the A/B rings and the long hydrocarbon chain of **18** and **71** and the benzyl group in **95** could be folded along the C/D rings of EMATE as depicted. This could be used to explain the high potencies observed for these compounds and that they might be acting in a similar manner to that of EMATE at the enzyme active site. *In vitro* studies have supported such a prediction as shown by the level of potency observed for these compounds. Even though the compounds in the 4-alkyl series show a steady increase in inhibitory activity as the chain length of alkyl group increase, the compounds in the 3-alkyl-4-methyl series are more potent for the same alkyl chain length in placental microsomal preparation. Although the series of compounds which have different substituents at the C-3 or C-4 positions showed good inhibitory activities, compound **95** was found to be the most potent compound with an IC_{50} value of 8 nM in placental microsomes.

It is likely that the best inhibitors of coumarin sulphamates are effective therapeutic agents and could be potentially development candidates for the treatment of HDBC,

whose clinical use would allow the role and potential of STS inhibitors in the treatment of postmenopausal women with HDBC to be fully evaluated.

CHAPTER 4

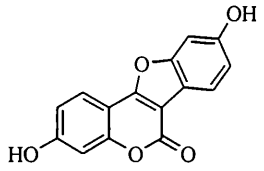
CHAPTER 4

Tricyclic coumarin sulphamates

4.0 Background

A need for non-steroidal inhibitors emerged due to the highly estrogenic nature of EMATE **(2-20)** (Figure 2.9, Chapter 2). The coumarin sulphamate derivatives with hydrophobic groups at the C-3 and/or C-4 position of the coumarin ring have revealed the potential to design extremely potent E1-STS inhibitors (Chapter 3). In addition, the encouraging biological activities obtained for the initial tricyclic COUMATEs (2.5, Chapter 2) has led to a further investigation on tricyclic coumarin sulphamates, which are presumably ABC ring mimics of EMATE.

Our interest in the coumarin system as the core structure for the design of non-steroidal STS inhibitors originated from the report of coumestrol being an estrogen mimic. Coumestrol **(4-1)**, which is a plant-derived tetracyclic coumarin compound initially isolated from alfalfa tree and red clover in high levels whose structure resembles that of the natural estrogens. It interacts with the ER and has been demonstrated to produce estrogen-like effects in a number of *in vivo* and *in vitro* models.[90] Many coumestrol-type compounds containing a basic amine have been shown to be selective estrogen receptor modulators (SERMs). Therefore, it was reasoned that simple bicyclic coumarin sulphamates might exhibit steroid sulphatase inhibition, but they themselves or their starting coumarins are unlikely to be estrogenic. This reasoning was thus proved by the potency observed for COUMATE **(2-66)** and its derivative 3,4-dimethylcoumarin-7-*O*-sulphamate **(2-69)** (Chapter 2).



Coumestrol
4-1

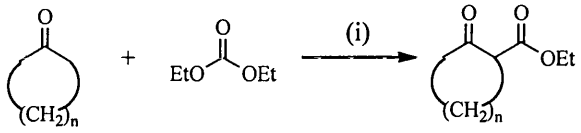
Figure 4.1 : Structure of Coumestrol

The idea of designing tricyclic COUMATEs as STS inhibitors emerged from the reasoning that the bicyclic coumarin moiety of COUMATE mimics the A/B ring system of EMATE. Incorporation of a third ring, which mimics the C/D ring of EMATE would be beneficial. The initial tricyclic compounds synthesised, such as 665 **(2-80)**, 666 **(2-81)**, 667 **(2-82)**, 668 **(2-83)** and 6613 **(2-84)** COUMATEs supported such reasoning by exhibiting highly potent E1-STS inhibitory activity in human placental microsomal preparation (Table 2.5, Chapter 2). 667 COUMATE in particular was found to be even more potent than EMATE *in vitro* with an IC_{50} value of 8 nM (c.f. IC_{50} = 25 nM for EMATE). 667 COUMATE is now being developed for Phase I clinical trials. To further improve the potency and also to investigate structure-activity relationship for this class of STS inhibitors several more tricyclic COUMATEs, with larger third ring sizes were synthesised in this project.

4.1 Synthesis

The starting cyclic β-keto esters required for the synthesis of tricyclic coumarin sulphamates beyond eight membered ring are unavailable commercially. Therefore, they were prepared by treating the corresponding cycloalkyl ketone with diethyl carbonate in the presence of two equivalents of sodium hydride (NaH) at room temperature[258] (Scheme 4.1). This synthetic method exploits the reasonably high acidity of the α-protons of a ketone, (pK_a = 10–14), which can be removed by NaH and by NaOEt. The carbanion that forms undergoes a nucleophilic attack on the carbonyl carbon of diethyl carbonate with the formation of the corresponding ethyl

2-oxocycloalkyl carboxylic acid ester. Dieckmann condensation is another known method for the synthesis of cyclic β-keto esters, in which the α carbon atom and the ester group for the condensation comes from the same molecule, i.e. intramolecular cyclisation. Though this is a valuable method, it is only useful for the preparation of five or six membered rings.



Scheme 4.1 : Synthesis of ethyl 2-oxocycloalkanecarboxylates (i) NaH, N_2, 15 h, R.T.

Keto-enol tautomerism is a feature of cyclic β-keto esters (Figure 4.2). The 9- and 10- membered ring containing β-keto esters exist in the enol form as well as the keto form. When keto and enol forms are in equilibria, the tautomers are slowly inter-converted at R.T. Such tautomerism was not observed for the 11, 12 and 15 carbon containing β-keto esters. This is presumably due relatively fast interchange that is taking place between the keto and enol forms. The proton signals of the individual isomers are clearly distinguishable in the NMR spectra of the 10 carbon containing β-keto ester, ethyl 2-oxocyclodecanecarboxylate **(120)** (Figure 4.3). The two sets of triplets corresponding to the CH_3 protons at δ 1.24 ppm and δ 1.31 ppm, and a set of quartets corresponding to the CH_2 protons of ethyl group at δ 4.13 ppm and δ 4.22 ppm were seen in a ratio of 1.2 : 1.8 and 0.5 : 1.5 of keto : enol.

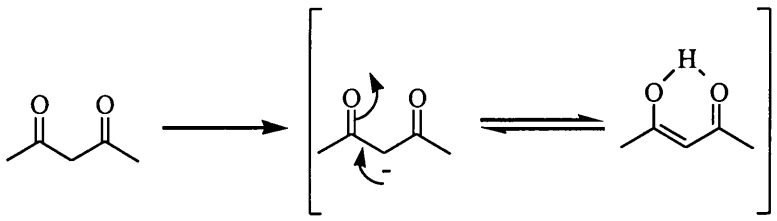


Figure 4.2 : Keto-enol tautomerism of the β-keto esters

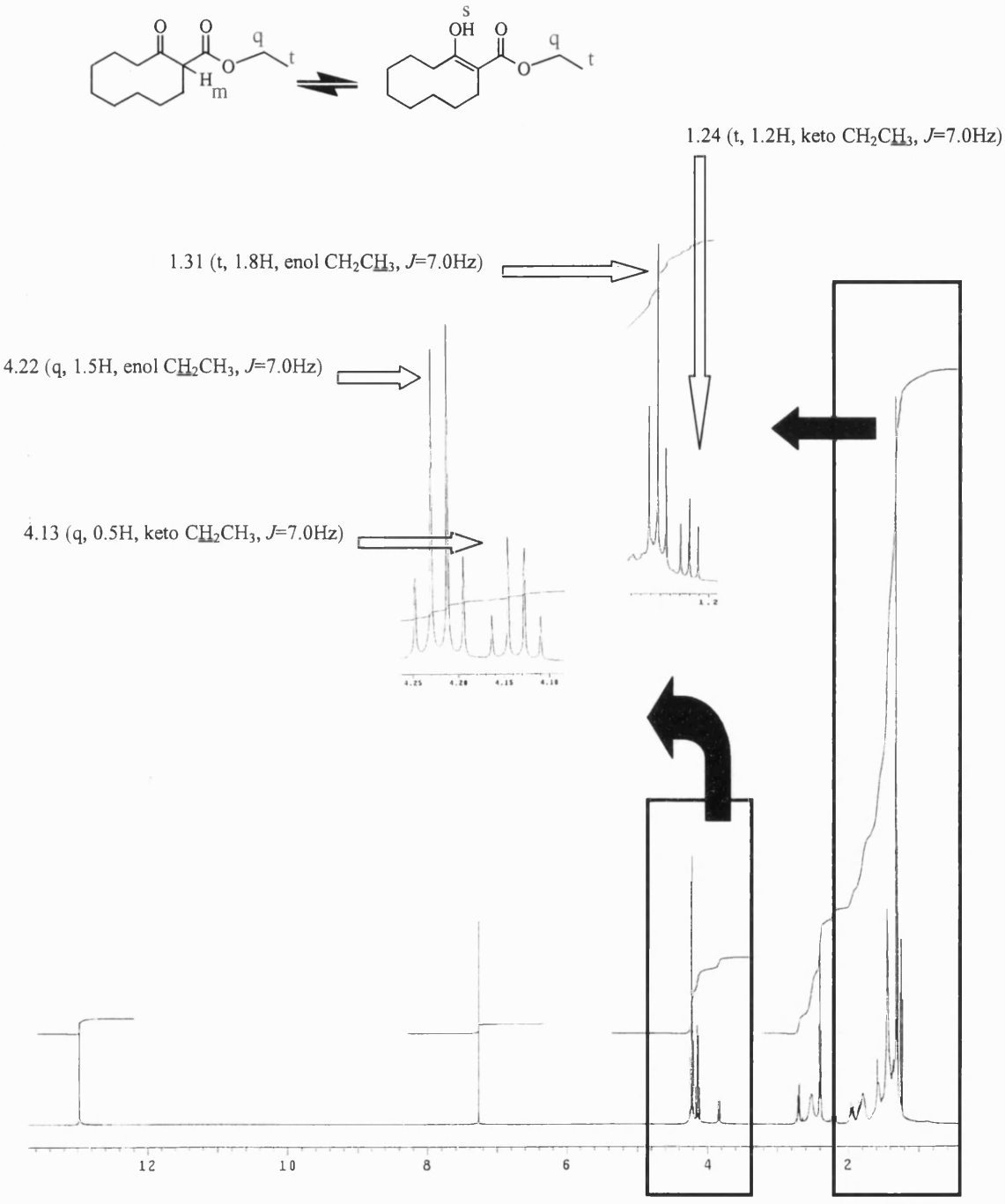
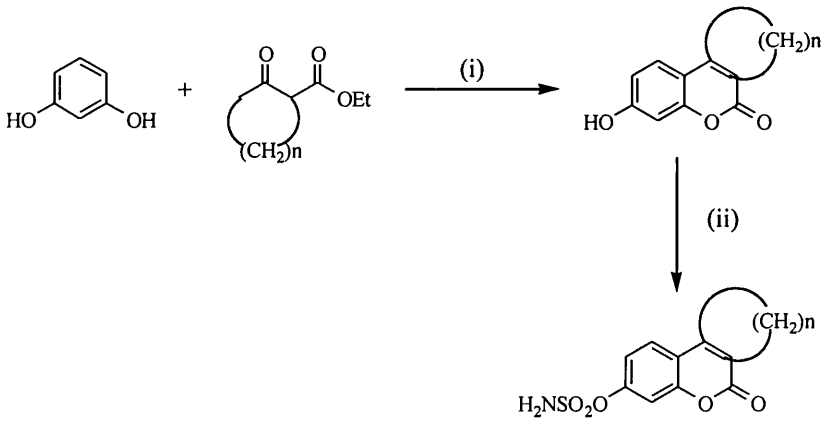


Figure 4.3 : NMR of ethyl 2-oxocyclodecanecarboxylate **(120)**

The strong deshielding effect of internal hydrogen bonding in the enolic form is well recognised (Figure 4.2) and it is generally accepted that the magnitude of the

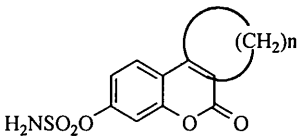
displacement of the OH signal to lower field is related to the strength of the hydrogen bond. The signal for the hydroxyl proton in the enol form was clearly visualised at δ 12.98 ppm for compound **120**. The signal for the α-hydrogen of the keto species was observed as a multiplet at δ 3.82-3.85 ppm.

The 9, 10, 11, 12 and 15 ring containing parent coumarins were formed under Pechmann reactions by cyclising resorcinol and the corresponding ethyl 2-oxocycloalkanecarboxylates in the presence of an equimolar mixture of trifluoroacetic acid (CF_3COOH) and conc. sulphuric acid (H_2SO_4) as the condensing agent. The yields of the tricyclic coumarins ranged from 14% to 33%. Such poor yields obtained were presumably due to the ring strain of the large cycloalkane rings mainly the 9- and 11-membered ring, which undergoes severe ring strain during cyclisation of the cyclic β-keto esters with resorcinol. The parent hydroxycoumarins formed were subsequently sulphamoylated with freshly prepared sulphamoyl chloride (Scheme 4.2).



Scheme 4.2 : Synthesis of tricyclic coumarin sulphamates (i) Conc.H_2SO_4/Conc.CF_3COOH, 3h, 0 °C to R.T. (ii) anhydrous DMF, NaH, N_2, 0 °C and H_2NSO_2Cl, 0 °C to R.T.

At the end of the sulphamoylation reactions the pure products were obtained only in lower yields between 30-50%. This is possibly due to the instability of the sulphamoylated compound. The weak O-S bond decomposes to its parent hydroxy compound. The unreacted starting materials were hence isolated. The COUMATEs became more and more difficult to separate due to increased lipophilicity as the size of the third ring got bigger. This is because the difference in the polarity between the parent hydroxy compound and the sulphamates progressively becomes smaller as the size of the third ring increases. As expected, recrystallisation of 6615 COUMATE proved to be difficult and it was obtained only as a waxy film.



		Compounds
119	DG 237B	669 COUMATE
122	DG 161B	6610 COUMATE
125	DG 209B	6611 COUMATE
128	DG 163B	6612 COUMATE
131	DG 213D	6615 COUMATE

Table 4.1 : Structures of the tricyclic coumarin-7-*O*-sulphamates

4.2 Results

The ability of the tricyclic COUMATEs to inhibit STS activity was evaluated. All the compounds synthesised in this series are potent inhibitors of E1-STS when tested in placental microsomes at various concentrations. (Table 4.2) The screening of some of the inhibitors at lower concentrations such as 0.01 μM and IC_{50} values are not yet available.

The biological activity of the tricyclic COUMATEs can be summarised as follows:

It was observed that the *in vitro* inhibitory activity was increased with the number of carbon atom in the third ring up to 6610 COUMATE, and from there onwards gradually decreased (Figure 4.8). All the compounds synthesised were found to be very active with inhibitory activities of >90% at 10 μM and 1 μM concentrations in placental microsomes. The inhibition ranges from 85% to 93% at 0.1 μM. It was found that 6610 COUMATE **(122)** is the most potent inhibitor *in vitro* with an IC_{50} value of 1 nM followed by 669 COUMATE **(119)** with an IC_{50} value of 2.4 nM. Surprisingly, contrary to what was observed *in vitro*, 6615 COUMATE **(131)**, which was the least active compound *in vitro* with an IC_{50} value of 370 nM was found to be highly potent *in vivo*.

		% Inhibition in placental microsomes				
		10 μM	**1 μM**	**0.1 μM**	**0.01 μM**	**IC_{50} nM**
119	669COUMATE	93 ± 1.5	91 ± 0.9	85 ± 0.2	*	2.4
122	6610COUMATE	95 ± 0.8	92 ± 0.3	88 ± 0.7	52 ± 5.5	1
125	6611COUMATE	93 ± 1	90 ±1	87 ± 1	*	13
128	6612COUMATE	99 ± 0.1	95 ± 0.6	93 ± 1.5	*	60
131	6615COUMATE	99 ± 0.1	97 ± 0.4	90 ± 0.6	*	370

Table 4.2 : Inhibition of E1-STS activity by the tricyclic COUMATEs in placental microsomes. * = Results not available.

The sulphamates synthesised were observed to be increasingly lipophilic as the third ring got bigger. Therefore, to further understand the relationships between the inhibitory activities and the hydrophobicity of tricyclic COUMATEs, log P values were determined using an HPLC analytical method and compared with those for the corresponding parent hydroxy compounds, estrone and EMATE **(2-20)**.

4.3 Log P calculations for tricyclic COUMATEs

Quantitative structure-activity relationship studies have shown that there is a relationship between the hydrophobicity of bioactive compounds and their biological activity. The absorption, distribution, metabolism and excretion (ADME) of a compound are greatly influenced by its lipophilicity. It is a generally held view that very lipophilic compounds are the preferred targets for metabolism, often leading to high clearance values. In addition, lipophilicity positively correlates with a high plasma protein binding. A large volume of distribution, probably due to a high fraction of the compound bound to tissues, is often observed for lipophilic compounds.

Usually, the hydrophobic property of a compound is expressed by the logarithmic value of the partition coefficient of the neutral form of bioactive compounds between n-octanol and water (log P_{oct}). The partition coefficient of a bioactive compound can be obtained from the following equation:

$$P = \frac{\text{Concentration of bioactive compound in octanol}}{\text{Concentration of bioactive compound in aqueous solution}}$$

Hydrophobic molecules will prefer to dissolve in octanol and have higher P values, whereas hydrophilic molecules will prefer to be in the aqueous layer and have lower values. Log P_{oct} is an indication that high lipophilicity (Log $P_{oct} > 4.5$) causes poor solubility. The shake-flask method is the standard method for determining log P_{oct}. However, determination by this method requires rigorous conditions to eliminate the effects of molecular association, acid dissociation and ion-pair formation between bioactive compounds and ions in the aqueous medium. This method forms emulsions that might produce false results. Therefore, centrifugation is necessary to separate the two phases and also this method requires relatively large amounts of pure compounds and octanol.[259] Furthermore, the exact determination of values below –2 and above 4 are very difficult and the whole

process is time consuming.[260] Therefore, a better and simple method for determining log P_{oct} is highly desirable.

Since the latter half of the 1970s, many attempts have been reported using thin layer chromatography (TLC), high performance liquid chromatography (HPLC)[261], dialysis[261(a)] and micro emulsion electro-kinetic chromatography[260] for determining log $P_{oct.}$ As a result, chromatographic methods in particular have been regarded as a useful means for determining log P_{oct} values, provided that they are employed carefully.

4.3.1 The procedure for determining the log P_{oct} values of tricyclic COUMATEs and other compounds by HPLC

The HPLC procedure described by Lombardo *et al*[261] was modified and used for the determination of the log P_{oct} values, which is now further discussed and explained in the context of how it was used.

First, a standard regression line between log k_w *vs* log P_{oct} was obtained for several compounds with known log P_{oct} values. For drug molecules, values calculated by the computer are often inaccurate and depending on the software used, they may differ by as much as two log P_{oct} units. Log P for the test compounds were calculated using CS Chem Draw Pro 5.0 software to obtain approximate values. (Table 4.3)

Compounds	Log P	Compounds	Log P
Estrone	4.40	6615 Coumarin	6.05
EMATE	3.73	COUMATE	*
Coumarin	*	665 COUMATE	1.21
665 Coumarin	1.88	666 COUMATE	1.63
666 Coumarin	2.30	667 COUMATE	2.04
667 Coumarin	2.71	668COUMATE	2.46

668 Coumarin	3.13	669COUMATE	2.88
669 Coumarin	3.55	6610COUMATE	3.29
6610 Coumarin	3.97	6611COUMATE	3.71
6611 Coumarin	4.38	6612COUMATE	4.13
6612 Coumarin	4.80	6613COUMATE	4.55
6613 Coumarin	5.22	6615COUMATE	5.38

Table 4.3 : Estimated log P values using CS Chem Draw Pro 5.0 software.

* : Values not available on CS Chem Draw Pro 5.0 software.

According to the computed values the log P ranges roughly from 1.20 to 6.00. Using these values as guideline twelve different drug molecules with known log P_{oct} were selected from the literature to cover the same lipophilicity range.[261]

Compounds	**Known log P_{oct}**	**Experimental log k_w**
Diethylstilbestrol	5.07	4.35
Nifedipine	3.17	2.36
Testosterone	3.29	2.81
Quinoline	2.03	1.33
3-Bromoquinoline	3.03	2.20
3,5-Dichlorophenol	3.68	3.08
Bifonazole	4.77	4.63
Clotrimazole	5.20	4.37
Tolnaftate	5.40	4.75
Estradiol	4.01	3.04
Diazepam	2.79	1.97
3-Chlorophenol	2.50	2.09

Table 4.4 : Known log P_{oct}, experimental log k_w and log P_{HPLC} values of the standard drug molecules used for the regression line. (S.D ± 0.01 and n = 3)

4.3.2 Experimental Procedure

In HPLC, the affinity of a sample compound for the stationary phase is characterised by the capacity factor k of the solute at a given mobile phase and k_w is the capacity factor extrapolated to a 0% concentration of the organic solvent in this case, methanol. k is expressed by Equation 1.

$$k = (t_R - t_0)/t_0 \quad (1)$$

t_0 and t_R are the retention times of an unretained reference compound and sample compounds respectively. Pure methanol was regarded as the reference compound, which can be detected at a wavelength of 210 nm. The retention time for pure methanol was determined to be 1.55 min. Injection of compound was repeated three times at each mobile phase. As mobile phase, 90 : 10, 80 : 20 and 70 :30 methanol and water was used. An average value was taken for each mobile phase as t_R and the corresponding k values were hence calculated from equation 1. The corresponding log k was calculated for each compound from the calculated k values. The log k_w was generated from the extrapolated regression line between mobile phase *vs* log k (Figure 4.4), which was obtained from the regression equation generated for each regression line, which is the intercept on the curve.[262]

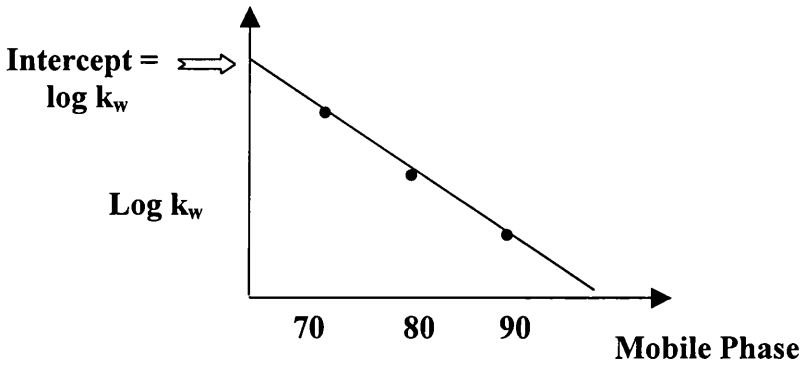


Figure 4.4 : A typical graph of mobile phase *vs* log k.

$$\log P_{oct} = a + b \log k_w \quad (2)$$

A linear standard calibration curve (Figure 4.7) was generated between log k_w and the known log P_{oct} values of the twelve drug molecules selected from the literature[261]. A regression equation was produced for the treadline using Microsoft Excel program, which is expressed by Equation 3.

$$\mathbf{y = 0.9495x + 0.8174} \qquad \mathbf{(3)}$$

Where,

$\mathbf{R^2 = 0.9619}$

The estimated slope of the final regression equation was within ± 0.05. R^2, which is the 'coefficient of determination' was also within ± 0.05 and this value was also calculated using Microsoft Excel program. R^2 is an indicator that ranges in value from 0 - 1 and reveals how closely the estimated values for the treadline corresponds to the actual data. The regression treadine is most reliable when its R^2 value is at or near 1.

The same procedure was used for the tricyclic COUMATEs and the parent hydroxy coumarins together with estrone and EMATE. The corresponding log k_w were calculated using the equations 1 and 2. Once the log k_w was calculated the log P_{HPLC} was obtained from the calibration curve generated for the standard drug molecules of known log P_{oct} either by directly reading off the regression line or calculated from the regression equation 3, where x = log k_w of the individual compounds (Table 4.5).

Calculated log k_w and log P_{HPLC} values

Compounds	Log k_w	Log P_{HPLC}
Estrone	2.97	3.64
EMATE	3.28	3.92
Coumarin	1.01	1.82
665 Coumarin	1.66	2.39

666 Coumarin	2.08	2.79
667 Coumarin	2.52	3.22
668 Coumarin	2.84	3.52
669 Coumarin	3.16	3.82
6610 Coumarin	3.62	4.26
6611 Coumarin	4.23	4.83
6612 Coumarin	5.08	5.64
6613 Coumarin	5.21	5.76
6615 Coumarin	5.71	6.24
COUMATE	1.01	1.77
665 COUMATE	1.61	2.34
666 COUMATE	1.99	2.71
667 COUMATE	2.46	3.15
668 COUMATE	2.79	3.46
669 COUMATE	3.09	3.75
6610 COUMATE	3.26	3.92
6611 COUMATE	4.19	4.80
6612 COUMATE	5.02	5.58
6613 COUMATE	5.18	5.74
6615 COUMATE	5.66	6.22

Table 4.5 : Experimental log k_w and log P_{HPLC} values obtained for the tricyclic hydroxy coumarins and COUMATEs compared with estrone and EMATE. (SD ± 0.02, n = 3)

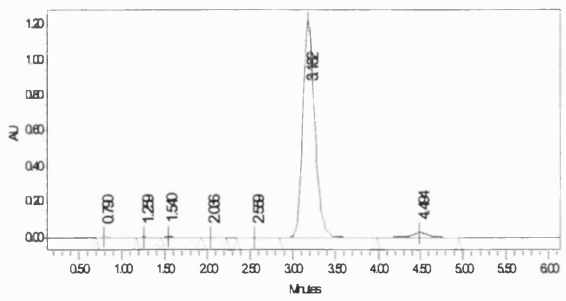
1 mg of each compound was dissolved in HPLC grade methanol and repeated three times at three different mobile phases, such as 90%, 80% and 70% methanol and the three points were extrapolated to the y-axis to give the k_w for each compound. All the chromatographic measurements were taken on a Waters HPLC, which

contains a reverse phase Waters Radialpak column (C18.8 × 100 mm) at room temperature without the use of octanol or any buffer. A number of studies indicate that a monolayer of methanol molecules is adsorbed on to the alkyl bonded stationary phase from MeOH-H_2O mobile phase, therefore it can be suggested that the stationary phase has a partial polar character and thus resembles n-octanol phase in octanol-H_2O systems implying that it is not necessary to use an octanol-coated column.[259]

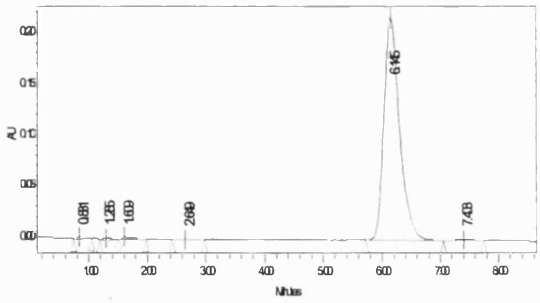
A diode array detector was used to monitor signals at 210, 230, 250 and 270 nm wavelengths and a flow rate of 2 ml/min was used. At each mobile phase the entire sample set was run before the column is equilibrated to the next condition. All statistical analyses were performed by Microsoft Excel program. Typical HPLC traces (Figures 4.5) and the graphs of mobile phase *vs* log k_w (Figure 4.6) corresponding to EMATE and 6610 COUMATE are given below. Detailed calculations of all of the compounds are included in the Appendix C.

4.3.3 Factors that affect log P_{HPLC}

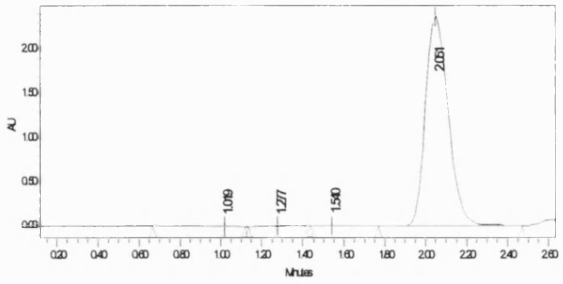
It should be noted that the log P determined by chromatographic methods is not the true partition coefficient of a compound, but a parameter that is related to the true partition coefficient, and there is no theoretical relationship between log k_w and log P_{oct}. Therefore, the partition coefficient calculated in this work is hence called log P_{HPLC}. However, this system is not suitable for determining high values of log P_{HPLC} because, increase of log P_{oct} by one log unit requires about ten times increase in t_R. The long elution times produce a broadening of the elution bands with inexact determination of the corresponding t_R values.



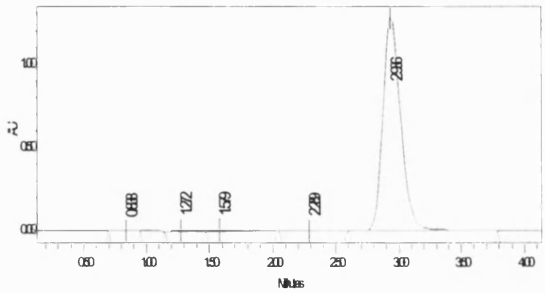
EMATE
(70:30 Methanol:Water)



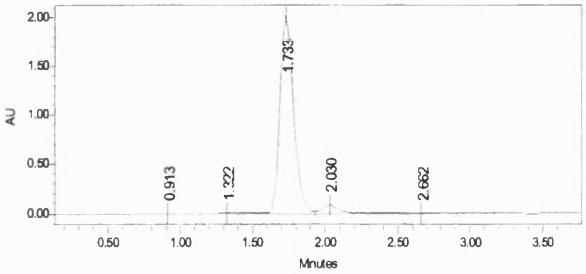
6610 COUMATE
(70:30 Methanol:Water)



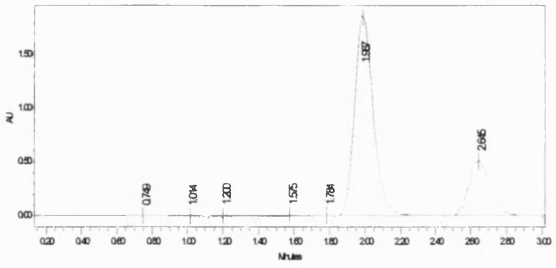
EMATE
(80:20 Methanol:Water)



6610 COUMATE
(80:20 Methanol:Water)



EMATE
(90:10 Methanol:Water)



6610 COUMATE
(90:10 Methanol:Water)

Figure 4.5 : Examples of typical HPLC traces of EMATE and 6610 COUMATE at different methanol : water concentrations.

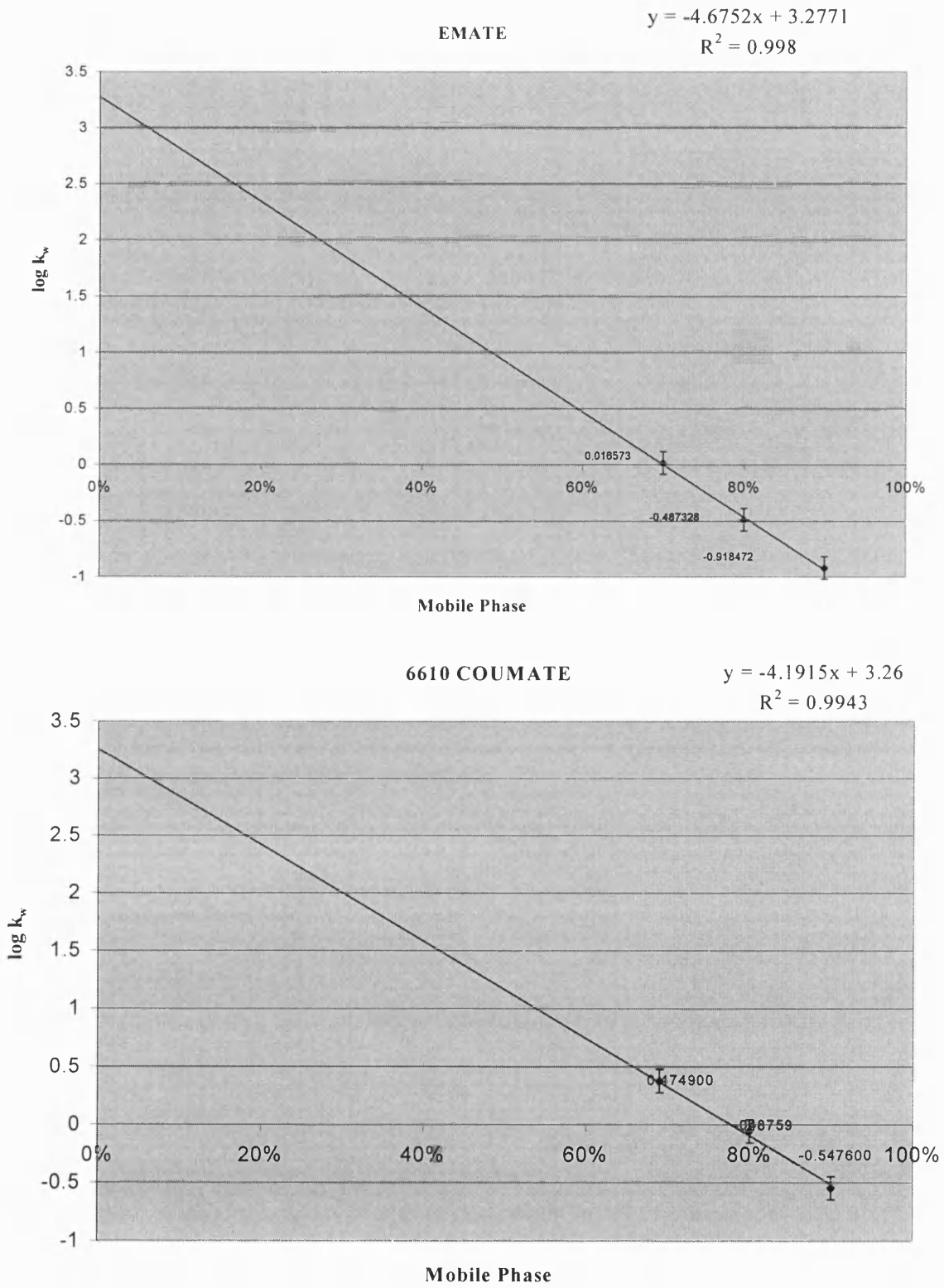


Figure 4.6 : Graphs of mobile phase *vs* log k of EMATE and 6610 COUMATE at different methanol : water concentrations generated by Microsoft Excel program.

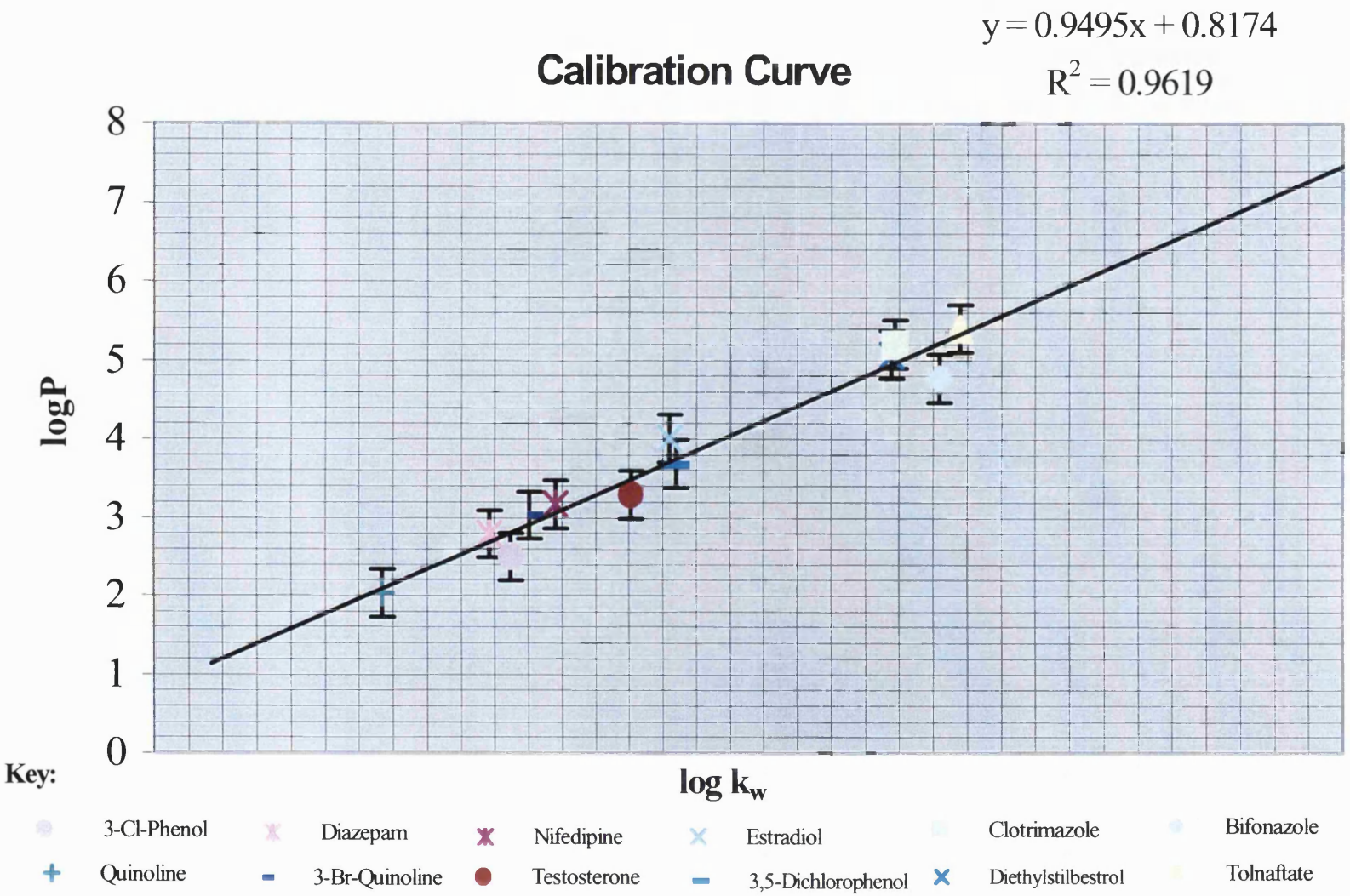


Figure 4.7 : Calibration curve generated for log k_w *vs* log P of compounds with known log P values

Other major factors that affect the log P_{HPLC} are[263]: (1) Temperature needs to be constant at all times. Here we have regarded room temperature to be the constant experimental temperature. (2) The nature of the stationary phase : t_R is directly related to the affinity of the test compound for the stationary phase, therefore the right choice of stationary phase is important. Generally, for a hydrophobic stationary phase such as octadecyl silica (ODS), n-octanol or a buffer (MOPS buffer)[261] is eluted through the column and stabilised, before the use of the test compound. Minick *et al* had used an MC-8 column coupled with octanol and a small amount of *n*-decylamine in the aqueous phase.[264,265] But, an electrostatically coated silica phase might not require a modifier such as *n*-decylamine. On the other hand, having octanol might be beneficial to reproduce the intermolecular interactions experienced by a solute in the 'shake-flask' method and might reduce the retention times slightly.[261]

However, we have ignored this factor and assumed the column (Waters Radialpack column) as having an 'octanol-like' property. Since the data obtained by Lombard *et al*, with and without octanol did not show a vast difference in the correlation, and gave a slope, which is close to unity in both instances, however, the errors are slightly higher in the absence of octanol. Equation 2 represents a linear free energy relationship in which the slope is an estimate of how closely the free energies of the processes compare. A slope close to 1 implies that the two processes have similar free energy changes. In this work, a slope of 0.9495 was obtained without the use of octanol. (3) The length and the diameter of the column must be the same through out the course of the measurements. We have used a fairly short column to enhance the throughput of the method (4) The composition of the mobile phase: In RP-HPLC, the mobile phase consists of buffering aqueous solution and a water-miscible organic solvent such as methanol, acetonitrile or tetrahydrofuran. We have chosen methanol and water of various concentrations to be the mobile phase. In general, log k decreases with increase in the concentration of the organic solvent in the mobile phase (5) The flow rate, which must be constant for all injections and we have used 2 ml/min for all measurements.

I have used a fairly acceptable number of standard compounds of 12 for the calibration curve (Table 4.4) with a good degree of accuracy across a wide range of lipophilicity values and hydrogen bonding properties. Considering all the assumptions and modifications done to the procedure described by Lombardo *et al*[261], the log P_{HPLC} values obtained are fairly accurate and within a range of ± 1.0 when compared to the computed values of these tricyclic compounds. We have demonstrated that this is a fairly accurate and rapid method for determining log P values with minimal manual intervention. Log P value of a compound is an important parameter to predict the solubility, permeability and clearance in the body. Therefore, the log P_{HPLC} values obtained could be used to explain the greater activity observed for the tricyclic compounds *in vitro* especially, the higher members of the series.

4.4 X-ray crystallography

X-ray crystal structures of 669 COUMATE and 6610 COUMATE were obtained in order to explore the conformation of the third ring in the crystal lattice and also to understand how it might bind to the enzyme active site. Suitable crystals of these compounds were obtained by slowly recrystallising from THF : hexane = 3:2. Approximate dimensions are of these crystals used for data collection are : 669 COUMATE **(119)** = 0.25 × 0.25 × 0.10 mm and 6610 COUMATE **(122)** = 0.30 × 0.20 × 0.20 mm. Measurements were made on a Nonius Kappa CCD diffractometer (Figures 4.8 and 4.9).

All three rings and the key features are clearly visible in the structures obtained. The asymmetric units of 669 COUMATE and 6610 COUMATE are seen to consist of a repeating unit of two molecules together with half a molecule of hexane is one of the solvents used for the recrystallisation. The molecules are linked together by intra/intermolecular hydrogen bonding involving NH_2 hydrogens. The geometric parameters for both molecules are similar within two estimated standard deviations.

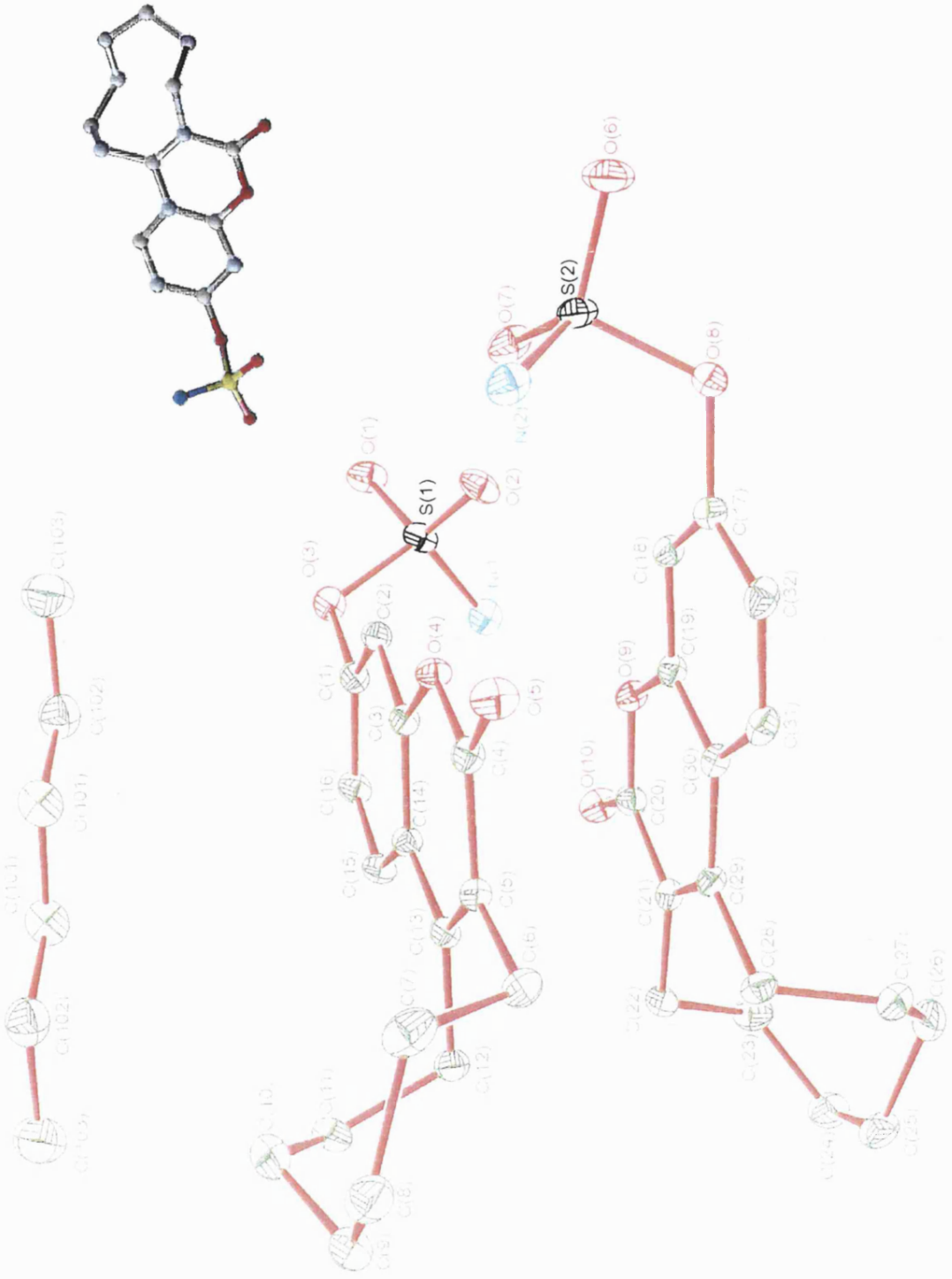


Figure 4.8 : X-ray crystal structure of 669 COUMATE **(119)**

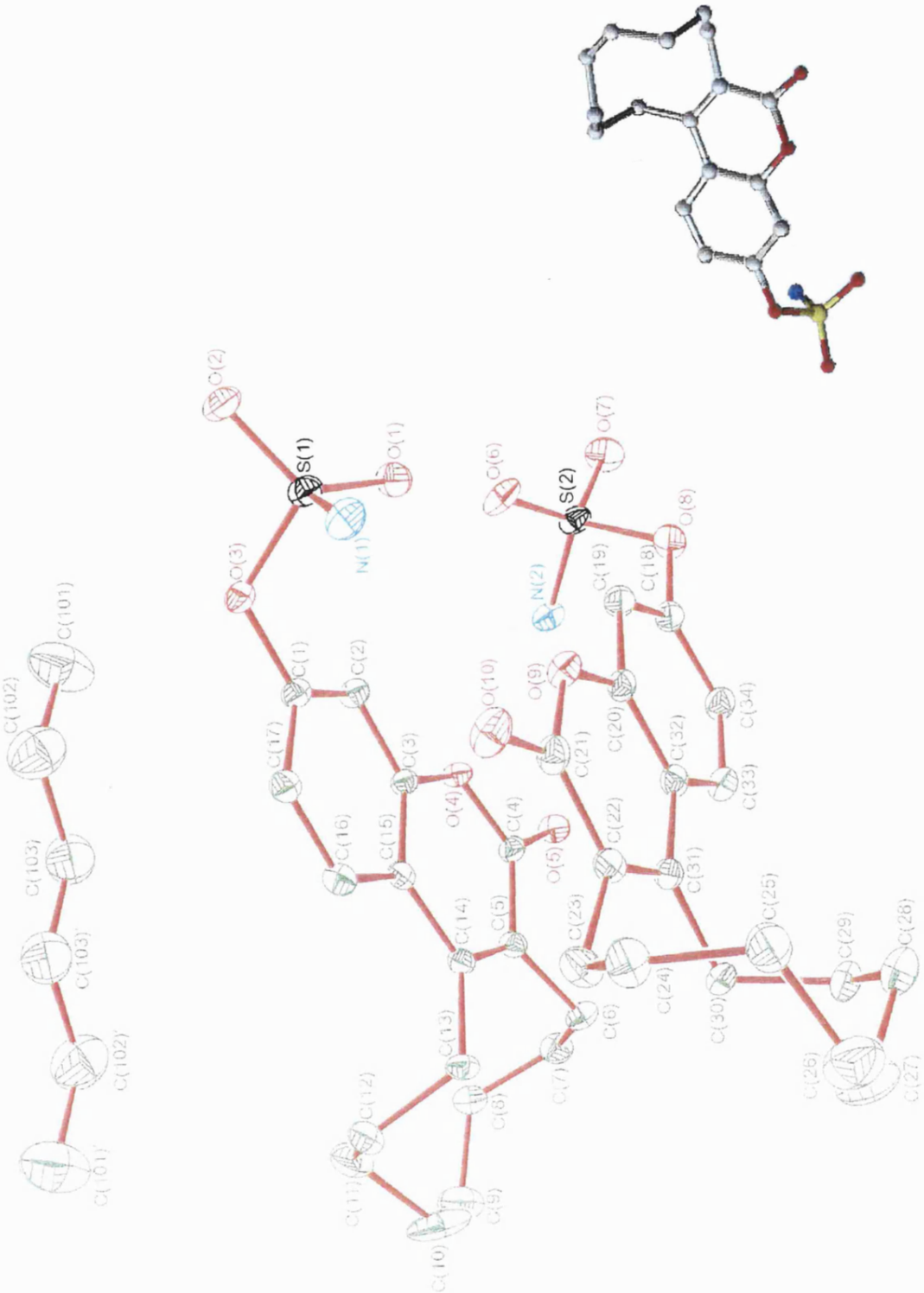


Figure 4.9 : X-ray crystal structure of 6610 COUMATE **(122)**

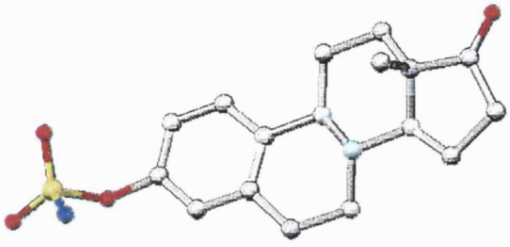
When looked through one of the sides of the crystals, it can be seen that the third cyclic ring folded in such a way that it exist in its lowest energy conformation. The aromatic ring is flat and rich in delocalised electrons, whereas the adjoining coumarin ring is relatively electron deficient due to the two electronegative oxygen atoms. Therefore, the two molecules form an interaction between their pi electron systems and hence stacked on top of each other to form pi molecular complexes.

From these X-ray crystal structures, the absolute conformation of 669 COUMATE and 6610 COUMATE molecules can be reliably determined. For 669 COUMATE, all C-C bond lengths were between 1.353 – 1.552 Å, all the C-O bond lengths were between 1.207 – 1.409 Å, all the S-O bond lengths were between 1.418 - 1.614 Å and the S-N bond lengths were 1.581 and 1.595 Å. The bond angles are in the range of 101.32 – 126.7 °. The symmetry transformation used to generate equivalent atoms : -x+1, -y, -z+1 through C(101). For 6610 COUMATE, all C-C bond lengths were between 1.361 – 1.597 Å, all the C-O bond lengths were between 1.215 – 1.413 Å, all the S-O bond lengths were between 1.4146 - 1.6141 Å and the S-N bond lengths were 1.576 and 1.587 Å. The bond angles are in the range of 102.43 – 126.1 °. The symmetry transformation used to generate equivalent atoms : -x+1, -y+1, -z through C(103). For full details, see Appendix D (1) and (2).

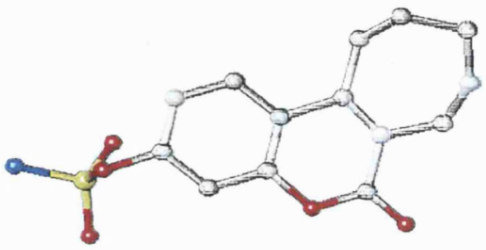
4.5 Discussion

4-Methylcoumarin-7-*O*-sulphamate (COUMATE) **(2-70)** was one of the first potent non-steroid based inhibitors identified.[196] It is possible that this 2-ringed compound might mimic the A and B rings of steroid based inhibitors, such as EMATE **(2-20)** (Figure 4.10). Therefore, it is reasonable to expect that a compound that mimics the A, B and C or D rings of EMATE or the steroid nucleus would be an even better inhibitor. Hence a series of tricyclic COUMATES (665, 666, 667, 668 and 6613 COUMATEs) were synthesised and tested. 665 COUMATE **(2-80)** was proved to be 4-times more potent than the 2-ringed COUMATE **(2-71)** with an IC_{50} of 200

nM,[219] (Table 2.4 and 2.5, Chapter 2) which suggested that such a strategy could lead to the development of more potent STS inhibitors than EMATE. The best compound was found to be 667 COUMATE **(2-82)** (Figure 4.10), whose IC_{50} of 8 nM in placental microsomes is some 3-fold better than EMATE (c.f. 25 nM). Such findings further validated our reasoning that inhibitory activity of a bicyclic coumarin sulphamate against STS can be increased by the incorporation of a third ring, which mimics the C/D rings of EMATE.

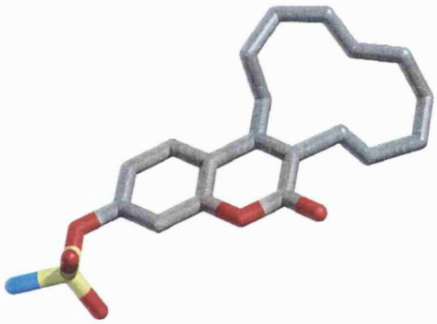


EMATE



667 COUMATE

6611 COUMATE



6612 COUMATE

Figure 4.10 : X-ray crystal structures of EMATE **(2-20)**, 667 COUMATE **(2-82)** and computer modelling pictorial representation of 6611 COUMATE **(125)** and 6612 COUMATE **(128)** on Sybyl®.

Of all the compounds synthesised in this series, 6610 COUMATE proved to possess the optimal size of the third ring for the binding to the enzyme active site

and hence was found to be the most potent STS inhibitor *in vitro*. 6610 COUMATE has an IC_{50} value of 1 nM, which is some 8-times and 25-times more potent than 667 COUMATE (c.f. IC_{50} = 8 nM) and EMATE (IC_{50} = 25 nM) *in vitro*. One possibility for the most potent STS inhibition observed with 6610 COUMATE might be that its third, 10-membered carbon ring folds in such a way that the interactions of the ring with those amino acid residues at the active site, which normally interact with the C and D rings of the steroid nucleus. The relatively sharp decline in potencies observed for 665 and 6615 COUMATEs has suggested that the interactions of their third ring with the amino acid residues in the enzyme active site are the least effective, either because it is too small or bulky. On the contrary, 669, 6610 and 6611 COUMATEs were all potent inhibitors with IC_{50}s of 2.4 nM, 1 nM and 13 nM respectively (Figure 4.11).

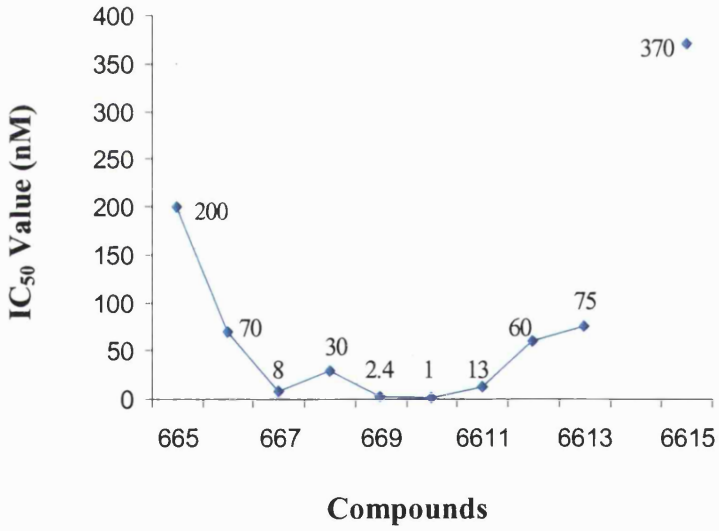


Figure 4.11 : Effects of the number of carbon atoms in the third ring of tricyclic coumarin sulphamates (665-6615 COUMATEs) on inhibition of STS activity in placental microsome preparation. The IC_{50} value for each individual inhibitor is shown on the X-axis.

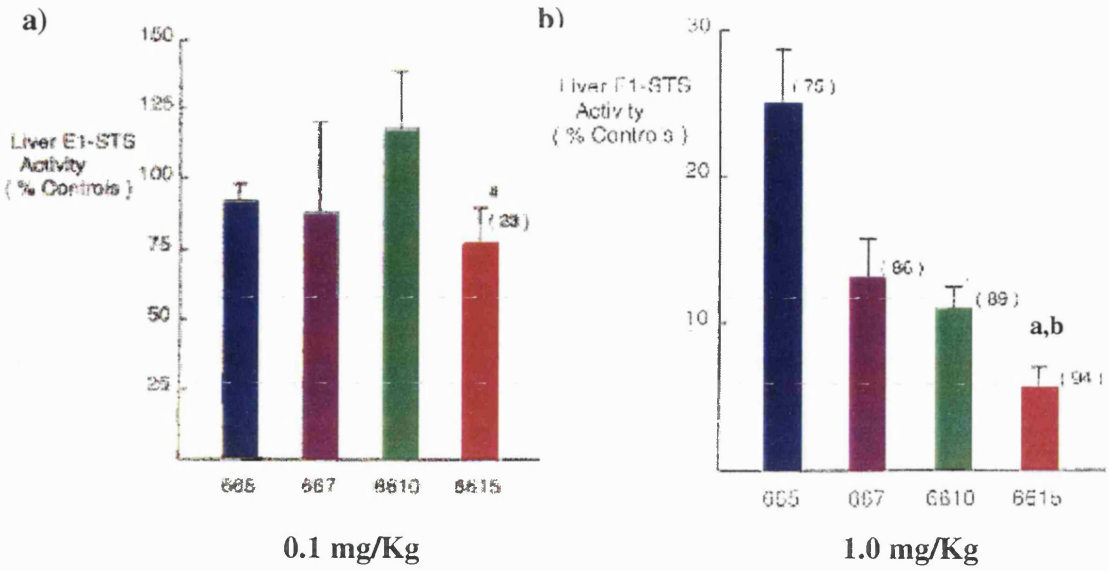


Figure 4.12 : *In vivo* inhibition of rat liver E1-STS activity by 665-, 667-, 6610- and 6615 COUMATEs. Female Wistar rats were treated with a single dose of vehicle, propylene glycol as control or the inhibitors at 0.1 mg/Kg and 1.0 mg/Kg. E1-STS activity was measured in samples of liver homogenate obtained 24 h after administration of the oral dose. Results are expressed as the percentage of remaining E1-STS activity compared with that in control animals (mean ± SD, n = 3). (a) 0.1mg/Kg; a, $p<0.05$ compared with controls. (b) 1.0mg/Kg; a, $p<0.001$ versus 667 COUMATE; b, $p<0.001$ versus 6610 COUMATE.

From the *in vitro* results, 6610 and 6615 COUMATEs were selected for *in vivo* studies. A single dose of 0.1 mg/Kg or 1.0 mg/Kg, p.o was administered to female rats. Unexpectedly, 6615 COUMATE with an *in vitro* IC_{50} value 370-times lower than that for 6610 COUMATE proved to be the most potent inhibitor *in vivo*. Of the series of compounds tested at 0.1 mg/Kg dose, 6615 COUMATE (**131**) was the only compound produced a small, but statistically significant ($p<0.05$) decrease (23%) in liver E1-STS activity. However, at 1.0 mg/Kg all the compounds tested significantly inhibited liver E1-STS activity (Figure 4.12). 667 COUMATE resulted in an 86% inhibition of E1-STS activity compared with that for animals receiving vehicle only. 665 COUMATE was less potent, achieving 75% inhibition

with 6610 COUMATE having a similar inhibitory potency to that of 667 COUMATE. Surprisingly, in view of its *in vitro* activity, the most potent compound *in vivo* was 6615 COUMATE inhibiting E1-STS activity by 94%. This is significantly ($p<0.001$) greater than the 86% inhibition achieved by 667 COUMATE at 1.0 mg/Kg. It is obvious that further studies are required to account for this apparent contradictory *in vitro* and *in vivo* inhibitory profile of 6610 and 6615 COUMATEs. Such findings are possibly due to species difference in STS since *in vitro* studies were carried out using human placental microsomes whereas for *in vivo* studies rat model was used (Figure 4.13). Also, the high lipophilicity of 6615 COUMATE as a result of its larger third ring might have profoundly affected the pharmacokinetics of the inhibitor *in vivo*.

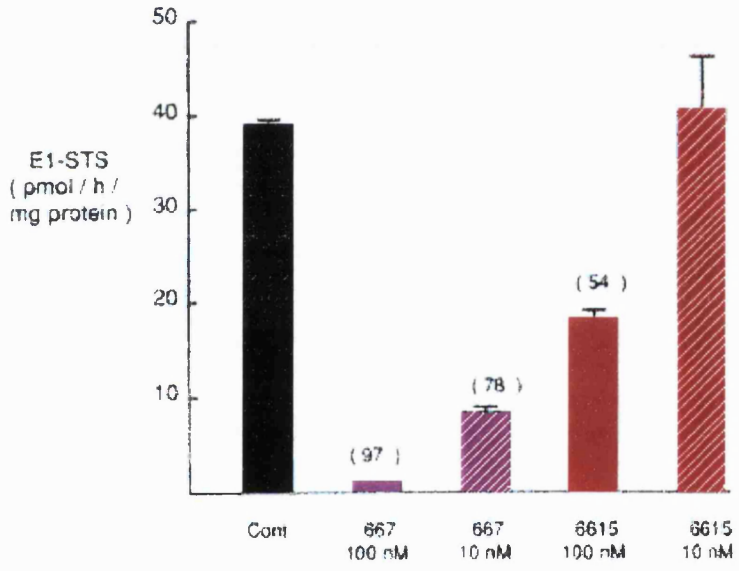


Figure 4.13 : *In vitro* inhibition of rat liver E1-STS activity by 667- and 6615 COUMATEs. (mean ± SD, n = 3). Figures in parentheses represent the percent inhibition compared with control animals.

There is now much evidence that in addition to the potential therapeutic roles in oncology and immunology, STS inhibitors may be of value in the treatment of

some disorders of cognitive function such as Alzheimer's disease.[198] Hence the ability of 6610 COUMATE, the best *in vitro* inhibitor in the series to inhibit brain STS activity *in vivo* was studied (Figure 4.14). Similar to what was seen previously for EMATE,[221] 6610 COUMATE also effectively inhibited STS activity by 76% in brain tissues at an oral dose of 0.1 mg/Kg. However, at a lower dose no significant inhibition of brain STS activity was detected.

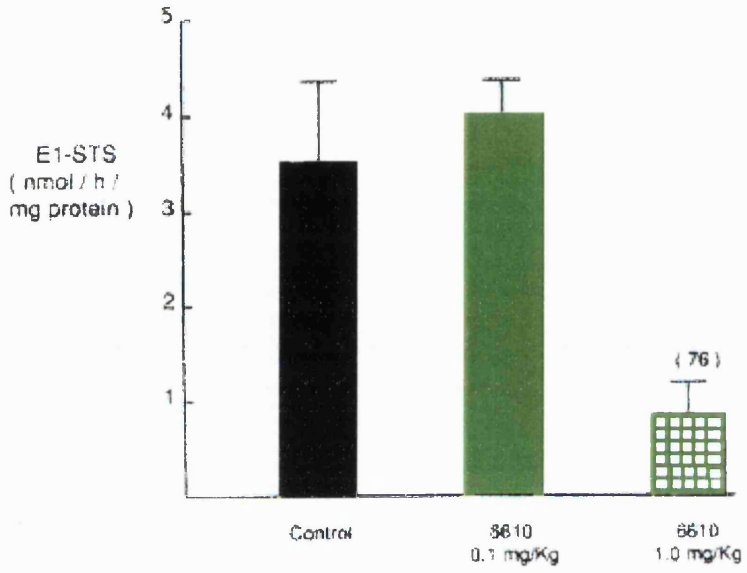


Figure 4.14 : Effect of 6610 COUMATE on rat brain sulphatase (*in vivo).* At the higher dose brain E1-STS activity was inhibited by 76% (means ± SD, n = 3). Dose : 0.1 and 1 mg/kg body weight, oral dose, 3 rats/dose, sacrificed after 24 hrs of treatments.

The inhibition of STS activity in white blood cells (WBC) was also examined with 6610- and 6615 COUMATEs (Figure 4.15) using the method developed previously[266] in which the extent and duration of STS inhibition was monitored. At 0.1 mg/Kg 6615 COUMATE inhibited WBC-STS activity by 45% whereas, 6610 COUMATE showed no effect. However, at a higher dose of 1.0 mg/Kg both compounds almost completely inhibited WBC-STS activity.

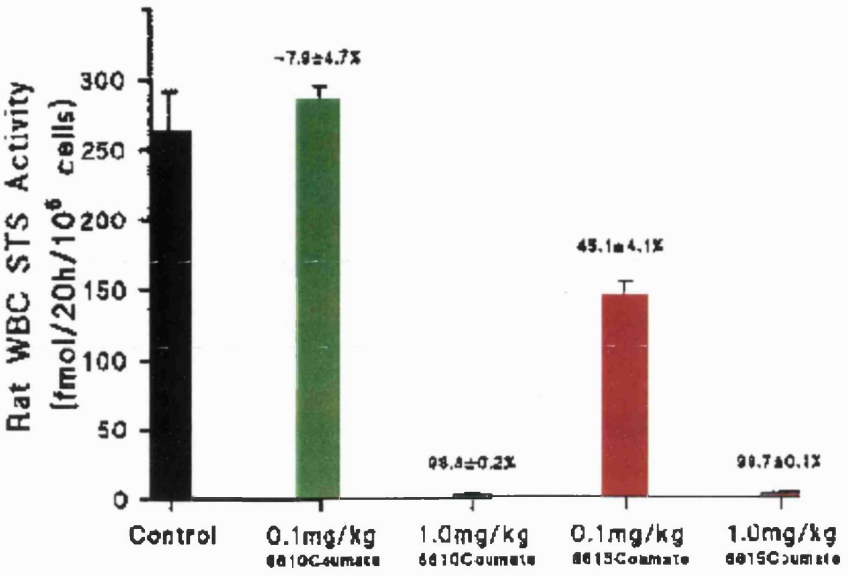


Figure 4.15 : Effect of 6610 COUMATE and 6615 COUMATE on E1-STS activity in white blood cells 24 h after administration (p.o.) of a single 0.1 mg/Kg or 1.0 kg/Kg dose (means ± SD, n = 3). Figures in parentheses represent the percent inhibition compared with control animals.

The log P values of these tricyclic COUMATEs have been determined by HPLC in order to establish if there is a correlation between the hydrophobicity of an inhibitor and its observed biological activity. According to the computed values the log P ranges from 1.88 to 6.05 for coumarins and 1.21 to 5.58 for COUMATEs. It can be observed that the log P values increase steadily as the third ring gets bigger and the log P value of the parent hydroxycoumarins are reduced when they are sulphamoylated. This trend was clearly observed from the log P values calculated for the corresponding parent compounds and the sulphamates (c.f. 6610 Coumarin = 3.97, whereas 6610 COUAMTE = 3.29) (Table 4.5).

The experimental log P_{HPLC} values ranges between 1.82 to 6.24 for the coumarins and 1.77 to 6.22 for COUMATEs. At least for the *in vitro* results, it appears that inhibitors whose potencies are better than or similar to that of EMATE have

experimental log P values in the range from 3.15 to 4.81 (Table 4.5). In particular, the log P_{HPLC} value obtained for 6610 COUMATE and EMATE are 3.92. With the experimental log P_{HPLC} value of 6610 COUMATE being similar to EMATE, despite the fact that the non-steroidal inhibitor is 25-fold more active, it is possible that the improved potency observed for 6610 COUMATE might be attributed to other contributory factors in addition to the hydrophobicity of the molecule, such as a greater sulphamoyl group transfer potential, i.e. lower pK_a of 6610 coumarin with respect to estrone.

The contradicting *in vitro* and *in vivo* activities of 6615 COUMATE (Calculated log P = 6.22, Table 4.5) are rather difficult to explain, since one would expect that a compound, which has a log P value greater than 5 to be less active. One of Lipinski's 'rule of 5' states that a highly lipophilic drug (log P > 5) is more likely to have poor solubility, absorption or permeability and would not be an active drug.[267] However, there are other factors, which contribute to the activity of a drug molecule, such as the nature of binding of the drug at the enzyme active site. 6615 COUMATE was assayed *in vitro*, in human placental microsomes preparation, whereas *in vivo* an animal model was used. Therefore, this difference in biological testing could be playing a major part in the observed difference in potencies *in vivo*. However, a definite explanation cannot be established unless further work is carried out.

The conformation in which the cyclodecene ring of 6610 COUMATE adopts upon binding to the enzyme active site might be such that it renders a very efficient interaction with those amino acid residues, which normally recognise the backbone of EMATE. The same reasoning can be applied to rationalise the relatively higher potency observed *in vitro* for 669 COUMATE (IC_{50} = 2.4 nM). Although the X-ray crystal structures of these compounds cannot show clearly the conformations these cycloalkene rings adopt on the enzyme, diagrammatically, it can be shown that the third ring in tricyclic coumarins could fold in such a way at the enzyme active site as to mimic the C/D rings of EMATE as depicted (Figure 4.16). The lower

potencies observed for 6611 and 6612 COUMATE are presumably due to their highly strained third ring, which might experience difficulty in forming the conformation like the steroid C/D ring at the enzyme active site hence, poor binding to the active site amino acids.

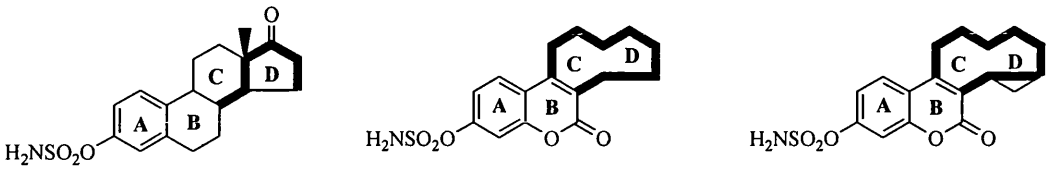


Figure 4.16 : Diagrammatic comparison of the folding pattern of the third ring in 669, 6610 and 6615 COUMATEs with EMATE.

The 17*β*-substituted carbamoyl **(2-56)** and the *N*-alkanoyl derivatives **(2-57)** of EMATE synthesised by Li *et al* are more potent inhibitors *in vitro*, but yet more lipophilic than EMATE[212] (Figure 2.13, Chapter 2). This could perhaps be because, Li *et al*'s compounds are exploiting an active site's hydrophobic pocket in the region of C-17 of EMATE and hence an increase in log P causes an increase in *in vitro* activitiy. However with the tricyclic COUMATEs, which are presumably ABCD ring mimics of EMATE, the log P values should be as close to that of EMATE as possible for them to be as potent as EMATE. This is exhibited by the calculated log P value of 6610 COUMATE (log P = 3.82), which is found to be similar to that of EMATE (Table 4.5).

4.6 Conclusion

We have further established in this work that tricyclic coumarin sulphamates represent a key lead template for the optimisation of potential STS inhibitors and highly potent non-steroidal, non-estrogenic derivatives can be produced by enlarging the third ring. In summary, the optimal size of the third ring for achieving the highest potency in a placental microsomes preparation is 10 as shown by the IC_{50} value for 6610 COUMATE, which was found to be 800-, 8- and 370 times

more potent than COUMATE, 667 and 6615 COUMATEs respectively. In the same assay, 6615 COUMATE was found to be the least potent inhibitor of the series (IC_{50} = 370 nM). However, upon evaluation of 665-, 667- and 6615 COUMATEs *in vivo* for the inhibition of rat liver E1-STS activity, 6615 COUMATE was found to be more potent (94% inhibition at 1 mg/kg dose) than 667 COUMATE (86%) (Figure 4.9), which is contrary to what would be expected from their *in vitro* activities. It is possible that the *in vivo* finding of 6615 COUMATE being the most potent inhibitor of STS activity could be due to the entry of 6615 COUMATE into tissues being facilitated by its higher lipophilicity, even though the log P of a bioactive drug should optimally be < 5. However, further studies are required to account for the differential *in vitro* and *in vivo* activities observed for 6615 COUMATE.

Whilst the most potent inhibition of STS *in vitro* was achieved with 6610 COUMATE, this inhibitor was found to be not as potent as its higher congener 6615 COUMATE *in vivo*. The X-ray crystal structures have revealed some interesting conformations of these non-steroidal compounds (Figures 4.8 and 4.9). The log P values of tricyclic COUMATEs might influence their biological activity, although it is unlikely that such property is the only contributory factor. These studies have identified a number of tricyclic COUMATES with therapeutic potential.

CHAPTER 5

CHAPTER 5

Indole Sulphamates

5.0 Background

Whilst all steroidal inhibitors of STS are primarily derivatives of EMATE **(2-20)** (Figure 2.9, Chapter 2),[187,221,224] and non-steroidal inhibitors apparently have more structural diversity than their steroidal counterparts. Sulphamates of single ring compounds, such as (*p-O*-sulphamoyl)-*N*-alkanoyltyramines[215] **(2-66)** and DES derivatives[190] **(5-1)**, bicyclic compounds such as THN sulphamates[190] **(5-2)** and coumarin sulphamates,[196,219,220]**(5-3)** tricyclic compounds such tricyclic COUMATEs[220,219] **(5-4)** (Chapter 4) and flavonoids[268] **(5-5)** are all STS inhibitors albeit to a different levels of potency (Figure 5.1).

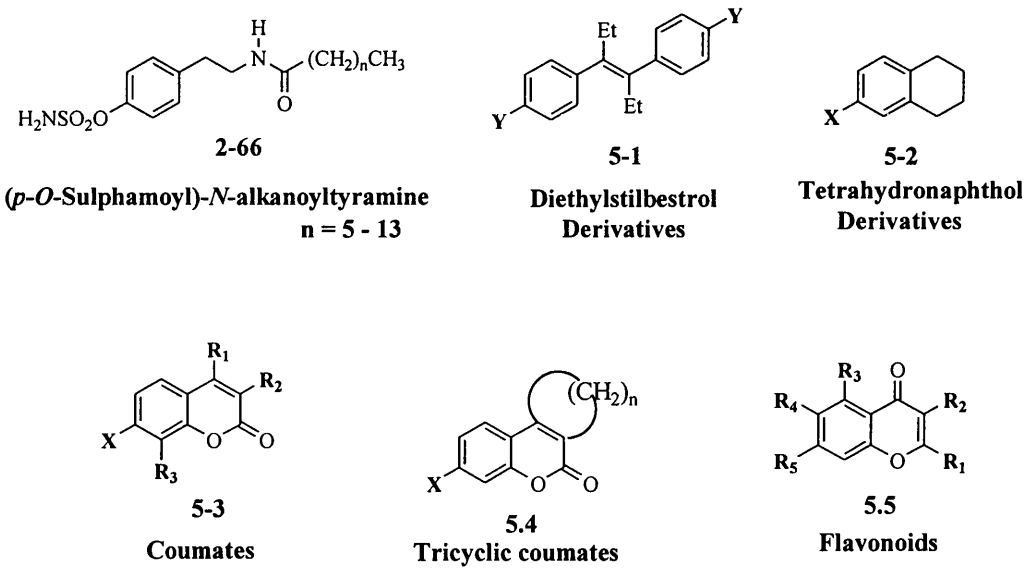


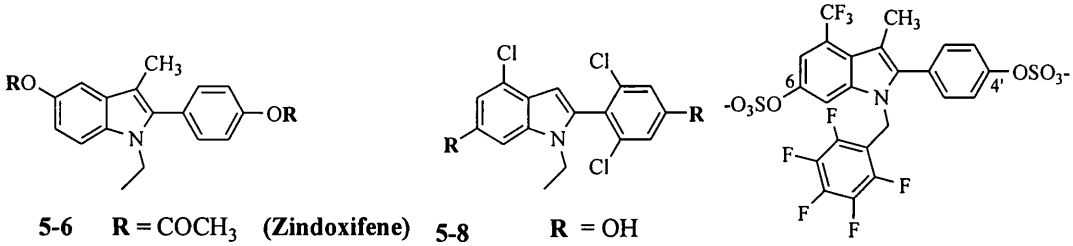
Figure 5.1 : Structures of the non-steroidal compounds explored so far

These compounds all share a common pharmacophore, which is a phenolic ring structure, another adjacent ring, a hydrophobic moiety attached to it and most importantly, the sulphamoyloxy group.[196] Although much success and progress

have been with coumarin sulphamates, it would be interesting to explore sulphamates of other structural moieties, which could also potentially be active against the STS enzyme.

Indole is an important heterocyclic system because it is built into proteins in the form of the amino acid tryptophan and also it provides the skeleton for many biologically active compounds and alkaloids. The incorporation of an indole moiety in biologically active compounds intended for endocrine therapy is not unprecedented. But, several indole containing compounds have been reported previously possessing antiestrogenic activities against mammary carcinomas[269,270] and also inducing angiogenesis and apoptosis.[271,272]

The work carried out by von Angerer *et al* identified a new class of mammary tumour inhibitors, the 2-(hydroxyphenyl)indoles.[269,270] The most promising compound of this class, 5-acetoxy-2-(4-acetoxyphenyl)-1-ethyl-3-methylindole (Zindoxifene) **(5-6)**, was shown to be metabolised substantially to its hydroxy conjugate **(5-7)** *in vivo*, which acts as a potential antiestrogen for the treatment of hormone dependent malignancies[273] (Figure 5.2). 1-Ethyl-4-chloro-2-(2,6-dichloro-4-hydroxyphenyl)-6-hydroxy indole **(5-8)** was found to have high affinity for the estrogen receptors and inhibit the growth of the 9,10-dimethyl-1,2-benz[a]anthracene (DMBA) induced mammary carcinoma of the Sprague-Dawley rat and showed uterotrophic activity and cytostatic effects against hormone-independent cells.[269]



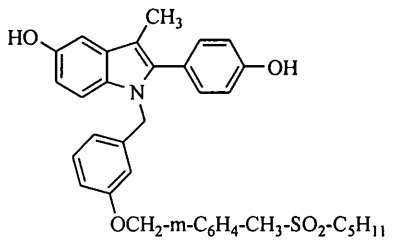
5-6 **R** = $COCH_3$ **(Zindoxifene)** **5-8** **R** = OH

5-7 **R** = H **5-9** **R** = OSO_3^- **5-10**

Figure 5.2 : Structures of indole containing compounds with anti-tumour activities

In subsequent work, Birnbock *et al* synthesised a number of mono- and disulphated derivatives of 2-(hydroxyphenyl)indoles to determine their sulphatase inhibitory activities.[274] Of all the derivatives synthesised, compounds **5-9** and **5-10** were the most potent, with IC_{50} values of 210 μM and 80 μM respectively (Figure 5.2). In these compounds, the presence of the two SO_4^- groups at positions C-6 and C-4' and the bulky hydrophobic group at the indole nitrogen are expected to be the features responsible for this marked increase in enzyme inhibitory activities.[274]

Extensive studies carried out by von Angerer *et al* on heterocyclic structures have demonstrated that side chains, characterised by a polar function such as an amide, or sulphoxide group linked by a polymethylene spacer group to the receptor binding moiety, determine the endocrine profile in respect to antagonism and potency. A number of 1-benzyl-2-phenylindoles and 1,2-diphenylindoles were synthesised and 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[3-[3-(pentylsulphonylmethyl)benzyl oxy]benzyl]indole **(5-11)** has now been identified as a potent antiestrogen[275] (Figure 5.3). It has been deduced that the existence of two hydroxyl groups in the molecule are prerequisite for a strong binding interaction with the estrogen receptor site. It is feasible that the methylene group, which acts as a hinge between the indole nitrogen and the phenyl group in the 1-benzyl-2-phenylindoles, mimics one of the tetrahedral carbon atoms in the steroid, giving rise to an orientation of the side chain similar to that of the steroidal pure antiestrogens.



5-11

Figure 5.3 : Structure of 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[3-[3-(pentylsulphonylmethyl)benzyloxy]benzyl]indole

When compared with the most popular antiestrogen tamoxifen, the indole compounds are superior at higher doses and are capable of reducing the initial tumour size while tamoxifen only delays the average tumour growth. The reduction of the average tumour size caused by the indole derivatives is due to the large fraction of the tumours regressing after administration.[270] When given in large doses, indoles accumulate in the target cells by binding to the ER and develop their antineoplastic activity *via* an estrogenic mechanism and non-specific cytostatic effects on hormone-independent mammary tumour cells. These indoles were also tested for their estrogenicity and antestrogenic activity *in vivo* and estimated in immature mice uterine weight test. It was found that the position of the oxygen functions and the size of the alkyl residues influence the character of the phenyl indoles and any minute alterations such as replacing the ethyl group with any other alkyl chain changes the biological profile of that compound. This is presumably due to the difference in conformational changes that occur in the drug-receptor complex.[270]

The finding that compound **5-10** is a substrate for the STS enzyme,[213,274] has suggested that its mono- or bis-sulphamate might potentially be an irreversible inhibitor of the enzyme. We observed a similar property with coumarin sulphate, which is a substrate for STS, and went on to develop coumarin sulphamates as time- and concentration dependent inhibitors of STS. Our initial approaches to the design of indole sulphamates as STS inhibitors were to sulphamoylate the indole ring at various positions, and investigate the inhibitory activities against STS. These investigations should provide a way for designing polycyclic indole sulphamates, with either fused or non-fused ring system in much the same manner as the development of coumarin sulphamates.

Therefore, to identify a new lead for a non-steroidal inhibitor and also as part of the on going programme to develop nonsteroidal inhibitors of E1-STS, indole ring system was chosen to explore further. Various indole sulphamate derivatives were hence synthesised with sulphamoyl group at the C-4, C-5 or C-6 positions of the

indole ring (Figure 5.4).

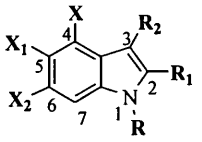
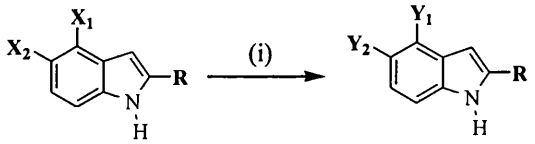


Figure 5.4 : General structure of the indole sulphamates synthesised. X = X_1 = X_2 =OO_2SNH_2, R = Alkyl chain and R_1 = H, alkyl or benzoyl group.

5.1 Synthesis

Since its first isolation in 1866 by Adolf Baeyer as a result of his studies on the degradation of indigo,[276] the synthesis of the indole ring system has captured the interest of organic chemists. Because of the potent biological activities exhibited by various indole derivatives, the methods of construction of this heterocyclic system have been subjected to considerable attention. Building an indole ring is well known. However, in this work, commercially available indoles were utilised to prepare the required indole sulphamates. The corresponding hydroxyindoles were deprotonated with NaH followed by an excess of sulphamoyl chloride furnished the desired indole-4-*O*-sulphamate **(132)**, indole-5-*O*-sulphamate **(133)** and 2-indolecarboxylic acid-5-*O*-sulphamates **(134)** in 39%, 32% and 49% yields, respectively (Scheme 5.1).



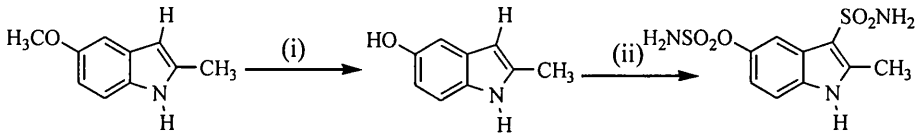
132 X_1 = OH Y_1 = OO_2SNH_2; X_2 = H Y_2 = H; R = H R = H

133 X_1 = H Y_1 = H; X_2 = OH Y_2 = OO_2SNH_2; R = H R = H

134 X_1 = H Y_1 = H; X_2 = OH Y_2 = OO_2SNH_2; R = CO_2H R = CO_2H

Scheme 5.1 : Synthesis of indole sulphamates (i) Anhydrous DMF, NaH, N_2, 0°C and H_2NSO_2Cl, 0°C to R.T.

2-Methyl-5-methoxyindole was demethylated with aluminium chloride ($AlCl_3$) to form the corresponding 5-hydroxyindole **(140)** in 35% yield. When compound **140** was sulphamoylated with an excess of sulphamoyl chloride using the usual procedure (Scheme 5.2), it was observed from the NMR spectra that the C-3 position of the indole ring was also sulphamoylated and gave **141** in a low yield of 17% (Figure 5.6).



140 **141**

Scheme 5.2 : Synthesis of compound **141** (i) $AlCl_3$, dry toluene, reflux, N_2, 3 h (ii) anhydrous DMF, NaH, N_2, 0°C and H_2NSO_2Cl, 0°C to R.T.

Indoles in general are very weak bases due to delocalisation of the nitrogen lone pair into the aromatic ring system. In a hydroxylated indole, the hydroxyl group at C-5 is more acidic ($pK_a \sim 10$) than the N-proton ($pK_a \sim 17$) therefore, deprotonation by 1 equivalent of NaH occurs almost exclusively at the hydroxyl group. Hence, the nucleophilic substitution by the sulphamoyl group readily takes place at the hydroxyl position first. Although *N*-sulphamoylation was not observed, it is possible that some deprotonation of the N-H also takes place. But, the resulting *N*-sulphamoylated compound is very unstable to isolate.

In addition to the desired sulphamoylation at the C-5 hydroxyl group of **140**, it was observed that sulphamoylation had also occurred at the indole C-3 position. Although this was disappointing it was not entirely surprising, because the C-3 position is the most highly electron-rich position in the indole system. Generating the oxyanion would also seriously activate the C-3 position and hence it easily undergoes electrophilic substitution at C-3 position to form a stable product

(Scheme 5.2).

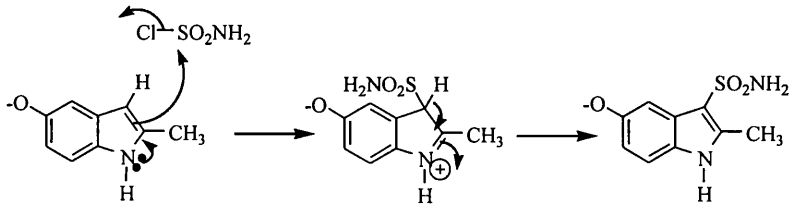
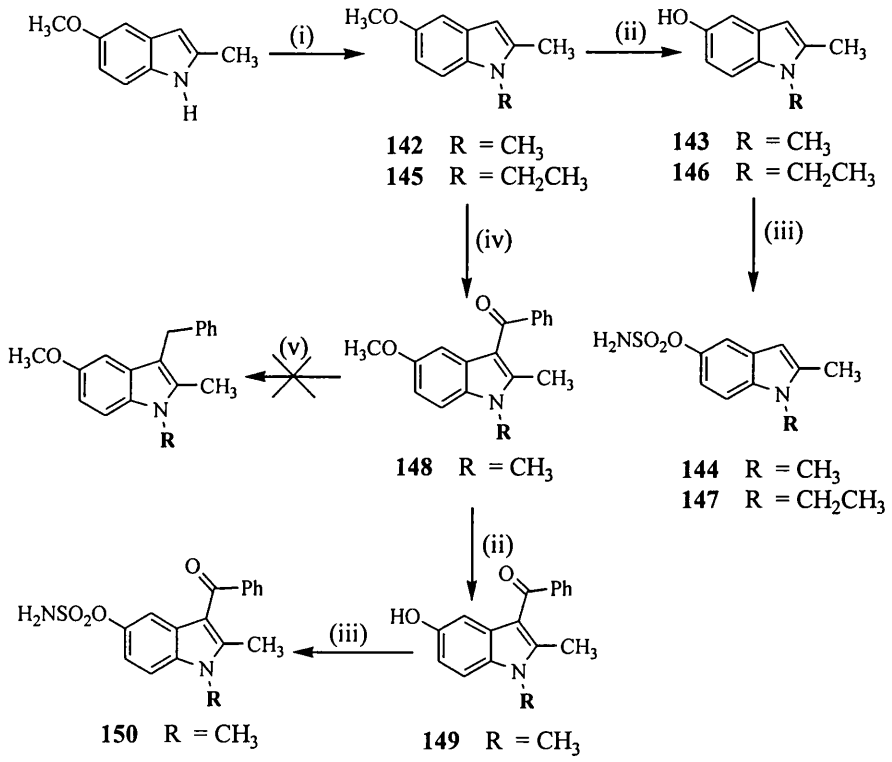


Figure 5.5 : Electrophilic substitution by the sulphamoyl group at the C-3 carbon.

Sulphamoylation at the C-3 and C-5 positions of the indole ring was observed in the corresponding NMR spectrum of compound **141**. The N-proton was clearly visible at δ 11.86 ppm, indicating that sulphamoylation had not occurred at this position. Deuteration causes a rapid exchange of the N-proton at neutral pH and also the four sulphamoyl protons, which were observed at δ 7.84 ppm. In the NMR spectrum of **141**, the C-3 proton signal, which appeared at δ 5.97 ppm for the compound **140** was absent. Such out come was not observed with compounds **132** and **133** which are unsubstituted at C-2 and C-3 positions, and also with compound **134**, which has a carboxylic acid group at the C-2 position. The presence of the electron-donating methyl group at the C-2 position of **141** must presumably be activating the C-3 carbon towards a nucleophile such as a sulphamoyl group.

Deprotonation of the indole nitrogen allows the introduction of a variety of alkyl groups by simple nucleophilic substitution reactions. To synthesise further derivatives of indole sulphamates, the N-proton was alkylated with methyl and ethyl groups. According to von Angerer, having a lipophilic side chain at the indole nitrogen shows a moderate to strong binding affinity for the estrogen receptor protein.[269] Therefore, the sodium salt formed from commercially available 5-methoxy-2-methylindole and NaH was alkylated with the corresponding alkyl iodide to produce 1,2-dimethyl-5-methoxyindole **(142)** and 1-ethyl-2-methyl-5-methoxyindole **(145)** in 22% and 68% yields, respectively (Scheme 5.3).

Although compounds **142** and **145** were the major products isolated, a small fraction of 1,3-alkylation product was also obtained during the synthesis of compound **145**. This probably due to subsequent alkylation of the highly electron-rich C-3 position of the N-alkylated indole with excess electrophile. The same could have taken place in the synthesis of compound **142**, which may explain the poor yield obtained for this reaction. Unfortunately, this cannot be confirmed since the impurities from this reaction could not be separated.



Scheme 5.3 : Synthesis of 2-methyl-1-alkylindole-5-*O*-sulphamate derivatives (i) NaH, DMF, RI, R.T. N_2, 3 h (ii) $AlCl_3$, dry toluene, reflux, N_2, 3 h (iii) anhydrous DMF, NaH, N_2, 0°C and H_2NSO_2Cl, 0°C to R.T. (iv) $AlCl_3$, CH_2Cl_2, benzoyl chloride, 3 h, R.T. (v) $NaBH_4/CF_3COOH$, CH_2Cl_2, 0°C to R.T., N_2, 12 h.

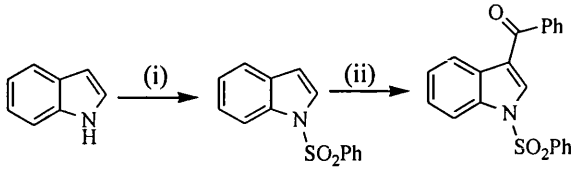
The *N*-alkylated indole derivatives were subsequently *O*-demethylated with $AlCl_3$ (Scheme 5.3) to form the corresponding 5-hydroxy derivatives 1,2-dimethyl-5-

hydroxyindole **(143)** and 1-ethyl-2-methyl-5-hydroxyindole **(146)** in 56% and 52% yields, respectively, before sulphamoylated with an excess of sulphamoyl chloride by the usual procedure. Clean reactions were observed and produced the desired 1,2-dimethylindole-5-*O*-sulphamate **(144)** and 1-ethyl-2-methylindole-5-*O*-sulphamate **(147)** as crystalline products in rather poor yields of 14% and 10%, respectively. Like what was observed with the compound **141**, sulphamoylation at the C-3 position was not noticed in these *N*-alkylated derivatives. This could be due to the fact that the alkylation at the N-H makes the C-3 carbon to be less reactive towards electrophiles. Hence, sulphamoylation takes place only at the deprotonated C-5 hydroxyl group.

Although the presence of alkyl substituents on the indole ring system has been reported to be useful for biological activity, having a group that could provide an enhanced binding to the enzyme active site would be highly desirable. From earlier investigations with coumarin sulphamates (Chapter 3), it was identified that having a phenyl or a benzyl group in the molecule is highly advantageous in terms of potent biological activity. It is also equally important to have these groups at the correct location on the ring. Therefore, it is likely that a similar approach to indoles also could provide an active compound. In the attempt to produce such compounds, approaches to the introduction of an acyl or benzyl groups at the C-3 position of the indole ring system were investigated.

Even though the addition of electrophiles to the C-3 position of the indole is the most characteristic reaction of this class of heterocycles and has been studied extensively, the synthesis of 3-acylindoles is often complicated by the fact that the indole displays ambident reactivity leading to competing substitution at the indole nitrogen. A previous attempt by Saxton mainly afforded 1,3-diacetylindole when a mixture of acetic anhydride and acetic acid was heated.[277] Reaction of indole magnesium salts with acid chlorides[278] or of Vilsmeier-Haack conditions involving dialkylamides and phosphorus oxychloride[279] produced only moderate yields of the desired products. These shortcomings can be defeated by acylation of the *N*-

protected compound using the Friedel-Crafts acylation procedure described by Ketcha and Gribble, where they have used phenylsulphonyl group to protect the indole nitrogen before acylating the 3-position with acetic anhydride or acid chlorides[280] (Scheme 5.4).



Scheme 5.4 : Friedel-Crafts acylation procedure described for indoles by Ketcha and Gribble using phenylsulphonyl protecting group.[280] (i) Benzenesulphonyl chloride (ii) $AlCl_3$, CH_2Cl_2, benzoyl chloride, 2 h, R.T.

Compound **142** was hence acylated at the 3 position of the indole ring using the Friedel-Craft acylation procedure. N-Protected indoles react readily with acid chlorides, therefore benzoyl chloride reacted at the C-3 position to yield 1,2-dimethyl-3-benzoyl-5-methoxyindole **(148)** in a poor yield of 13% (Scheme 5.3). Subsequent demethylation of compound **148** with $AlCl_3$ gave the 5-hydroxyindole **(149)**, which was sulphamoylated with excess sulphamoyl chloride to furnish 1,2-dimethyl-3-benzoylindole-5-*O*-sulphamate **(150)** in moderate yield of 32% (Scheme 5.3).

The reduction of the carbonyl group in the benzoyl substituent of compound **148** to the corresponding benzyl derivative **III** was found to be unsuccessful with the reagent, sodium tris(trifluoroacetoxy)borohydride **(I)** that formed between $NaBH_4$ and CF_3COOH (Figure 5.6). The product obtained contained a lot of byproducts, which were inseparable by flash chromatography. Plausible explanations could be that the highly electrophilic indole **(II)** formed (Figure 5.6) after the reduction with **I** is susceptible to attack by any surrounding nucleophilic species and hence forming several by-products.

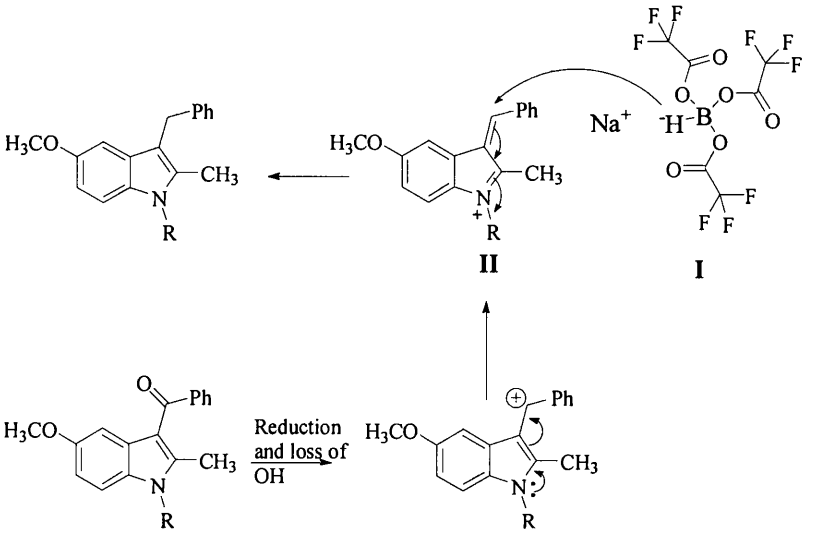


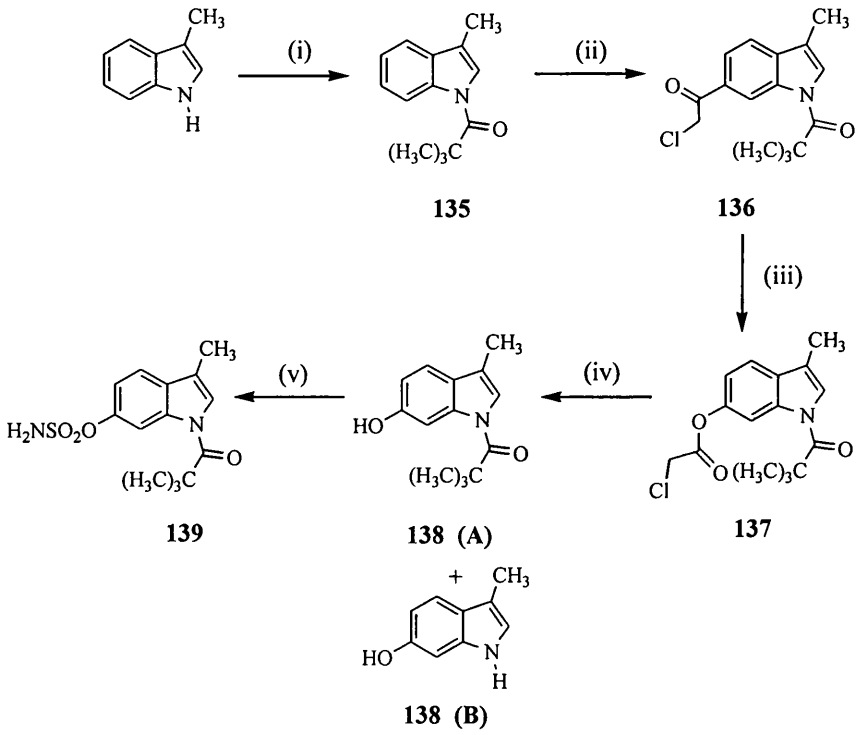
Figure 5.6 : Proposed reduction of compound **148** with **I** to form the product **III**

Having synthesised compounds with a sulphamoyloxy group at the C-5 position of the indole, the next task was to develop a method to synthesise compounds, with sulphamoyloxy group at C-6 position. This is to investigate the change in biological activity by placing the sulphamoyl group in a different position of the indole system. It has been reported that 6-hydroxyindoles possess a range of interesting pharmacological activities and are valuable precursor to some important indoles and indole alkaloids.[281]

Substituted 6-hydroxyindoles have been synthesised previously by various methods, from alkyl tyrosinates and N-bromosuccinimide,[282] and from 4-hydroxybenzaldehyde and methyl α-azidoacetate.[283] There are also several other reactions, such as Meerwein arylation, which was reported by Raucher *et al*,[284] involving a diazonium salt derived from an appropriate 2-nitroaniline. Other methods postulated by Feldman *et al* using 4-methoxy-2-nitrotoluene, condensed with *N,N*-dimethylformamide dimethyl acetal,[285] Reissert indole synthesis[286] and, more recently, the Leimgruber-Batcho method[276] are also possible routes to

synthesise 6-hydroxyindoles. But they are comparatively lengthy, involve cyclisation from simple starting materials, cumbersome and gave poor yields.

Direct hydroxylation of the benzene ring of indole derivatives was reported to occur in the reaction with hydrogen peroxide in super-acids, but regioselectivity is poor.[287] Therefore, the reaction described by Teranishi *et al* was chosen to carry out because the desired 6-hydroxyindole is relatively easily prepared from corresponding commercially available indoles in rather good yields[281] (Scheme 5.5). Also, this elegant regioselective oxidation of substituted indoles is reported only once before by Teranishi *et al.*[281]



Scheme 5.5 : Synthesis of 3-methyl-1-pivaloylindole-6-*O*-sulphamate **(139)** (i) NaH, $(CH_3)_3CCOCl$, DMF, 0°C, 1 h (ii) $AlCl_3$, $ClCH_2COCl$, CH_2ClCH_2Cl, R.T. 3 h (iii) *m*-CPBA, Na_2HPO_4, CH_2Cl_2, R.T. 1 h (iv) $NaSCH_3$, CH_3OH, R.T. 1 h (v) anhy. DMF, NaH, N_2, 0°C and H_2NSO_2Cl, 0°C to R.T.

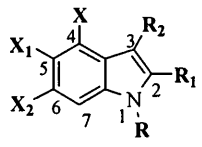
The anion formed by the deprotonation of the NH proton of 3-methylindole with NaH was acylated with pivaloyl chloride to produce the 3-methyl-1-pivaloylindole **(135)** in 77% yield (Scheme 5.5). The bulky pivaloyl group hence sterically protects the C-2 position from any unwanted subsequent reactions. The regioselective acylation of compound **135** at the C-6 position with $AlCl_3$ and chloroacetyl chloride gave 6-chloroacetyl-3-methyl-1-pivaloylindole **(136)** in 48% yield, without the formation of 2-chloroacylated product (Scheme 5.5). Only disadvantage of this reaction however was the use of a large excess of $AlCl_3$ (4.5 equivalents) and chloroacetyl chloride (4.7 equivalents) in 1,2-dichloroethane was used, which was a very rigorous reaction to do when carried out in a large scale. Also, **135** must be added slowly to the $AlCl_3$/chloroacetyl chloride complex in order to minimise any by-product formation.

In the literature, oxidation of indole derivatives with peracid,[288] or singlet oxygen[289] has always taken place at the pyrrole ring of indole. The reason being that the pyrrole ring is more nucleophilic than the benzene ring, which prevents the direct oxidation on the benzene part. Therefore, a regioselective method to introduce an oxygen function at the C-6 position is highly desirable. Baeyer-Villiger oxidation with *m*-chloroperbenzoic acid (*m*-CPBA) was chosen for our purpose since it has been reported to furnish selective oxidation at the C-6 position of the indole in high yield in a similar reaction.[281] Hence, compound **136** was treated with *m*-CPBA in CH_2Cl_2 at R.T. in the presence of anhydrous sodium hydrogen phosphate ($NaHPO_4$), which acts as a base to obtain 6-chloroacetoxy-3-methyl-1-pivaloylindole **(137)** in 72% yield (Scheme 5.5). There were no reaction observed when $NaHPO_4$ was omitted or non-anhydrous $NaHPO_4$ was employed with the recovery of the starting material **136** as a result.

The hydrolysis of the 6-chloroacetate group of **137** to its phenolic derivative and the deacylation of the N-atom can be carried out simultaneously using different basic reagents, such as, potassium carbonate/methanol or sodium hydride/methanol, but these reagents have reported to be very low yielding.[281] Therefore, the hydrolysis

and deacylaton reactions were carried out by stirring **137** for 2 h with sodium thiomethoxide in methanol at R.T. and under a N_2 atmosphere (Scheme 5.5). Unfortunately, the main product obtained was 6-hydroxy-3-methyl-1-pivaloylindole **(138 A)** in a very low yield and had the pivaloyl group still attached to the nitrogen atom (Scheme 5.5). The desired product **(138 B)** was only a little fraction, which was recognised in the crude product isolated from flash chromatography and was not attempted to purify any further. Since the pivaloyl group is more hindered for the thiomethoxide anion to attack at R.T., optimising the conditions of this reaction such as, having an excess of sodium thiomethoxide or stirring the reaction mixture for longer or raising the temperature could produce the desired product as the major compound. The major **(138 A)** and the minor **(138 B)** products were identified from the NMR spectra, where the peak corresponding to the nine protons of the pivaloyl group remained at δ1.51 ppm, which was also supported by mass spectral analysis. Compound **138 A** was subsequently sulphamoylated by the usual procedure using an excess of sulphamoyl chloride to furnish 3-methyl-1-pivaloylindole-6-*O*-sulphamate **(139)** in 24% yield.

The following compounds were synthesised in the indole sulphamate series:



	R	R_1	R_2	X	X_1	X_2
132	H	H	H	OO_2SNH_2	H	H
133	H	H	H	H	OO_2SNH_2	H
134	H	CO_2H	H	H	OO_2SNH_2	H
139	$COC(CH_3)_3$	H	CH_3	H	H	OO_2SNH_2
141	H	CH_3	H	H	OO_2SNH_2	H
144	CH_3	CH_3	H	H	OO_2SNH_2	H
147	CH_2CH_3	CH_3	H	H	OO_2SNH_2	H
150	CH_3	CH_3	H	H	OO_2SNH_2	H

Table 5.1 : Structures of the indole sulphamates

5.2 Results

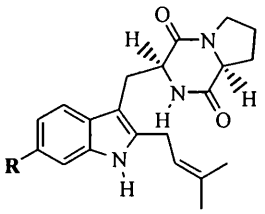
The ability of the indole sulphamate derivatives to inhibit E1-STS activity was examined in MCF-7 breast cancer cells and in placental microsomes (Table 5.2). The biological activity data for all the compounds are not available at present.

5.3 Discussion

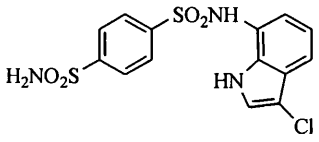
During the course of this work, substituted and unsubstituted indole sulphamates with the sulphamoyloxy group at the C-4, C-5 and C-6 were synthesised.

An alkyl substituent at the indole nitrogen helps for a strong binding with the ER protein. Derivatives with hydrogen in position 1 of the indole ring do not bind to the receptor, presumably because they form hydrogen bridges with water molecules. Therefore, the polar moieties formed prevent the necessary hydrophobic interaction with the receptor site.[270] The most favourable structures are those with a methyl or ethyl group at 3-position of the indole nucleus and an ethyl or propyl group on the nitrogen. Two contrary effects can rationalize this maximum affinity: the favourable increase in lipophilicity in the centre of the molecule and the steric hindrance by larger groups.[270]

Recently, it has been reported that some indole-containing derivatives play a crucial role in cell proliferation, differentiation, transformation and apoptosis.[271,272] (See Chapter 6 for detail) Malignant tumour cells can be clearly distinguished from normal cells by their chaotic proliferation, due to a serious disorder in their cell cycle regulatory mechanism. Cell cycle inhibitors or modulators that halt uncontrollable tumour growth are regarded as highly promising new therapeutic agents against human cancers. Tryprotatins A **(5-12)** and B **(5-13)**, which were first isolated as secondary metabolites of a fungal marine strain BM939 were shown to inhibit completely the cell cycle progression of tsFT210 cells in the G2/M phase at a concentration of 50 μg/mL of **5-12** and 12.5 μg/mL of **5-13** respectively[272] (Figure 7.5, Chapter 7).



5-12 Tryprostatin **A** R = OCH_3
5-13 Tryprostatin **B** R = H



***N*-(3-chloro-7-indolyl)-1,4-benzenedisulphonamide**
5-14

Figure 5.7 : Structures of some promising indole derivatives with clinical potential

Another promising drug that has been recently reported is *N*-(3-chloro-7-indolyl)-1,4-benzenedisulphonamide **(5-14)**, the indole derivative of previously known antimitotic agent E7010 (Figure 7.5). This drug inhibited the cell cycle progression of P388 murine leukemic cells in the G1 phase unlike its lead compound, which blocked the G1/M phase. This indole sulphonamide showed a significant antitumour activity against HCT116 human colon carcinoma in both *in-vitro* (IC_{50} = 0.11 μg/mL in cell proliferation assay) and *in vivo* also showed a marked reduction of tumour size in nude mice. Because of this excellent activity, this compound is currently undergoing Phase I clinical trials in Europe.[271] Therefore, as well as exhibiting E1-STS inhibitory activities, it is very much likely that the indole derivatives synthesised in this project would also likely to possess antimitotic or apoptotic properties. Unfortunately, the nature of these of inhibition of these compounds can only be validated once the biological activity results are made available, which is currently underway.

5.4 Conclusion

In conclusion, a series of indole sulphamate analogues have been prepared and their biological evaluation is ongoing at present. Indole sulphamates may presumably be effective therapeutic agents in the treatment of estrogen-dependent breast cancer as well as possessing potential antimitotic and apoptotic activities. These results would lead also to further studies to design and maximise the activity of

metabolically stable and potent E1-STS inhibitors and to make the compounds completely devoid of estrogenicity, as it known that some indole derivatives are non-estrogenic.[269]

CHAPTER 6

CHAPTER 6

Microtubule Disruptors

6.0 Introduction

Following heart disease, cancer is the biggest cause of death in the West. Cancer is a generic term for over 200 diseases, which share a number of characteristics including uncontrolled cellular proliferation. This uncontrolled growth can impinge on surrounding organs, causing disruption of normal bodily functioning, which in turn can lead to death. Another feature of cancer is the ability of tumour cells to migrate to other sites in the body. This process (metastasis) also increases the difficulty in treating these diseases as those secondary tumours can also disrupt bodily functions. Under these conditions, the removal of tumours by surgery becomes less practicable and other methods of treatment are needed such as chemotherapy.

Cancer chemotherapy is designed to exploit the differences between normal and malignant cells. Thus the ultimate goal of chemotherapy is to produce a drug, which will specifically destroy, or otherwise render benign, cancer cells without having a significant effect on the normal cells. Cancer cells differ from their normal counterparts in a number of biochemical processes, particularly in the control of cell growth and cell division. Inhibiting such key cellular processes in the tumour can cause the cancer cells to regress. Angiogenesis and apoptosis are some of these processes in cancer that can be exploited in controlling tumour cell proliferation. However, these differences can be small as a result of alterations or damage to only a minute proportion of normal cellular processes and hence the design and synthesis of a truly selective anticancer drug to target specifically these cellular processes of the cancer has yet to be accomplished.

6.1 Angiogenesis and diseases

Angiogenesis is a process by which new blood vessels are formed. In healthy adults, angiogenesis occurs during menstrual cycle, wound healing, fetal development and numerous pathological processes. Disrupting the angiogenesis cascade is a novel approach to the treatment of cancer, heart disease, several forms of blindness, psoriasis, and many chronic inflammatory diseases. The market for this approach is significant to date, since the existing therapies are largely inadequate. It is forecasted that angiogenic therapeutics could represent a vast pharmaceutical market with sales in excess of $3,770 M by the year 2005 and the value of the angiogenic oncology market alone is estimated to be $3 billion in the same time frame.[290]

6.2 Angiogenesis and cancer

Under homeostatic conditions, angiogenesis is a highly regulated process and is essential for tumour progression and the formation of metastases. The endothelial lining of a blood vessel is normally quiescent. The vascular endothelial cells that form blood vessels rarely proliferate. In cancer the switch from resting state to the angiogenic state occurs when homeostasis is altered by pathological processes, such as tumour growth. Under such conditions, angiogenesis is stimulated by a change in the net balance between pro- and anti-angiogenic factors. The angiogenic switch can be triggered by an increase in pro-angiogenic factors or a decrease in anti-angiogenic factors. Additionally, several studies have further indicated that up-regulation of angiogenic stimulators is often accompanied by a down-regulation of local tissue inhibitors of angiogenesis.

6.3 Tumour growth and metastasis

All solid tumours are composed of two distinct but inter-related compartments: the malignant cells themselves and the tissue framework or stroma, in which the tumour cells grow. A major compartment of this stroma is the vascular supply, which is required for the malignant progression. Rapid and exponential tumour growth requires ongoing angiogenesis. It appears that rapidly growing tumours

actively influence the angiogenic switch to sustain continuous cell proliferation. In the absence of angiogenesis, tumour growth is limited to 1-2 mm^3. Growth beyond this size requires the angiogenic phenotype. Expansion of tumour mass is made possible not only through perfusion of blood but also through paracrine stimulation of tumour cells by numerous growth factors and matrix proteins produced by the new capillary endothelium.

Angiogenesis is also an essential component of the metastatic pathway. These vessels provide the principle route by which tumour cells exit the primary tumour site and enter the circulation. It has been shown that highly vascularised malignant tumours have a higher incidence of metastasis than poorly vascularised malignant tumours. Moreover, once tumour cells have metastasised, their sustained growth is once again dependent upon establishing a new blood supply.

6.4 Angiogenesis therapeutic strategy

Tumour angiogenesis is essential for tumour progression and the formation of metastasis. In the adult, ~0.01% of endothelial cells undergo cell division. By contrast, the fraction of proliferating endothelial cells in tumours is proposed to be 50-fold higher.[291] These differences between proliferating endothelial cells in tumour tissue *vs* normal tissue can be exploited through the use of angiogenesis inhibitors. Furthermore, a single tumour vessel may supply as many as 10^4 tumour cells, thereby amplifying the anti tumour effects of antiangiogenic compounds.[291] Importantly, tumour endothelium is derived from normal host cells and in contrast to tumour cells, is genetically stable, which suggests that tumour endothelium is unlikely to develop resistance to cytotoxic agents.[292] Recent investigations have focused on the development of antiangiogenic agents, particularly those that can be administered p.o. and for prolonged period, as alternatives to standard cytotoxic anticancer therapies.

Angiogenic therapies may act either directly by interfering specifically with the ability of endothelial cells to form new capillary blood vessels or indirectly by influencing the microenvironments that regulate tumour angiogenesis. There are multiple pharmacological targets in this process currently under evaluation for therapeutic potential. Investigations of antiangiogenic compounds have been conducted preclinical and clinical trials. Strategies to inhibit angiogenesis have included the use of neutralising antibodies to angiogenic proteins, integrin molecules[293] and growth factor receptors.[294] Also, kinase inhibitors, natural products such as TNP-470 and antibiotic derivatives such as minocyclin have been used.[295] Angiostatin and endostatin, enzymatic degradation products of plasminogen and type XVIII collagen respectively, are reported to induce tumour regression and prolong tumour dormancy in murine model systems. However, in most cases, tumour re-growth ensues after cessation of treatment.[296] Tumour cures have been limited when most angiogenesis inhibitors are used as a sole method of treatment. Angiogenesis inhibitors represent a promising cancer therapy for several reasons: Effective angiogenic agents should have wide applicability as a general anticancer therapy as tumours of many diverse histological types must induce angiogenesis to grow beyond a size of 1 to 2 mm^3 and the angiogenic pathway used is generally independent of tumour type.

Drug resistance is a severe limitation of conventional chemotherapy. The emergence of acquired drug resistance depends in part on the genetic instability, heterogeneity and high mutational rate of tumour cells. In contrast, endothelial cells are genetically stable, homogeneous and have a low mutational rate. Angiogenic therapy directed against tumour-associated endothelial cells should induce little or no drug resistance. To date, studies with angiogenesis inhibitors, such as rhAngiostatin, have shown no drug resistance in animal studies. In addition to the potential in the monotherapy of cancer, angiogenic agents can be used in combination with traditional chemotherapy and radiotherapy. To date, promising results have been achieved using angiogenesis inhibitors in combination with traditional cancer therapies.

6.5 Apoptosis

The control of cell number and the removal of inappropriate or damaged and potentially dangerous cells are critical both in development and for adult tissue homeostasis. This regulation occurs *via* coordinated control of cellular proliferation, cellular differentiation and cell death. Apoptosis or programmed cell death is a widespread physiological process that has steadily gained recognition due to its clearly established roles in tissue development, tissue homeostasis and disease pathogenesis. This is an active mode of cellular suicide, which is encountered among normal as well as tumour cells in physiological and pathological situations. When there is an abnormal proliferation of cells taking place in the body, apoptosis is induced by specific cellular processes, and those abnormal cells are effectively removed from the body to protect from any harmful effects. Therefore, a drug that could induce apoptosis would be useful as an anticancer agent.

Cell death was understood to be an element in normal development as early as 1951[297] and good experimental evidence that it was integrated into developmental programmes was provided by Saunders[298] in 1966 and Lockshin and Beaulaton[299] in 1974. In 1971, John Kerr first introduced the concept of apoptosis during his studies to define the role of lysosomes in lethal hepatocyte injury.[300] Kerr observed single cells that appeared to be dying, but he saw no evidence of dispersion of their lysosomal enzymes. Extensive studies revealed this unique process of cellular cell death and in 1972 they named this process apoptosis.[301,302]

The apoptotic process involves an orchestrated cleavage of DNA and organelles including the nucleus and mitochondria, together with an activation of signals for phagocytic engulfment and destruction. The damaged cells with mutated DNA are removed from the body by apoptosis. This prevents the proliferation of malignant clones or the propagation of cells containing viral DNA.[303] *In vivo*, apoptotic cells are phagocytosed by macrophages. *In vitro*, however, they are not phagocytosed, so that they can last until the apoptotic process is completed.

The morphology of apoptosis is well characterised. Deregulation of the apoptotic pathway can lead to diseases, most notably cancers, autoimmune diseases[304] and neurodegenerative disorders such as Alzheimer's disease.[305] In breast cancer, an altered balance between the rate of survival and proliferation contributes to the increased cell number seen in neoplasia. In the breast, secretory epithelial cells are removed extremely rapidly by apoptosis and in rodents mammary glands can completely be remodeled within a few days.[306] The role of apoptosis in a wide variety of diseases, together with its highly regulated nature, has made it an attractive new target for therapeutic intervention in diseases previously considered incurable.

6.6 Mechanism of cell division

In 1903, Boveri suggested that cancer might arise as a consequence of chromosome gain or loss. Many subsequent studies have backed up this insightful proposal and put this theory in the centre stage.[307] The microtubule system plays an important role in eukaryotic cells and is an attractive target for the development of anticancer agents. A cancer can be viewed, as a condition where the delicate balance between cell production and cell death is lost, resulting in an overproduction of cells. This increase in cell growth and division presents an attractive and achievable target for drug design.

A typical cell cycle is divided into two phases: Mitosis and Interphase. Mitosis is the stage in the process of cell division where segregation of chromosomes occurs prior to cell replication. It has been recognised for a century as the mechanism in eukaryotes for partitioning the genetic material (DNA) equally at cell division. This process occurs in a normal cell undergoing division and thus does not represent a truly specific target. This division of the nucleus is usually followed immediately by cytokinesis, the division of cytoplasm. The basic sequence leading to cell division is well established. Microtubules, dynamic pipe-like protein fibres, are highly dynamic organelles that are essential in mitosis.[308] Chemicals that attack microtubules through tubulin, their major structural component, disrupt or suppress

both microtubule structure and normal functions by inhibition or promotion of microtubule assembly. This action results in cell arrest at mitosis.[309]

The microtubules function vitally for cell support, by acting as a form of internal scaffold, giving the cell both shape and an organised structure, cellular transport by moving the cell around its environment and transporting organelles around the cellular interior. However, arguably the most important role of the microtubules is the formation of the mitotic spindle, which is intimately involved in cell replication, one of the most complex and demanding processes undertaken by the body[310] (Figure 6.1).

During cell division, the cell must completely duplicate its internal components, including the whole of its DNA, so that it can form two identical daughter cells. The cell then orders the DNA to split into two identical sets of chromosomes and separate them into two distinct parcels at opposite ends of the cell, ready to form the two nuclei in the daughter cells. This ordering and relocation of the genetic material, which takes about an hour, is known as mitosis. Thus a gap of time occurs after DNA synthesis and before cell division and another gap was found to occur after cell division and before the next round of DNA synthesis. This analysis led to the conclusion that the eukaryotic cell cycle consists of 4 phases: an M (mitotic) phase, a G1 phase (the first gap), the S phase (DNA synthesis), a G2 phase (the second gap), and back to M. The phases between mitoses (G1, S and G2) are collectively known as the interphase (Figure 6.2).

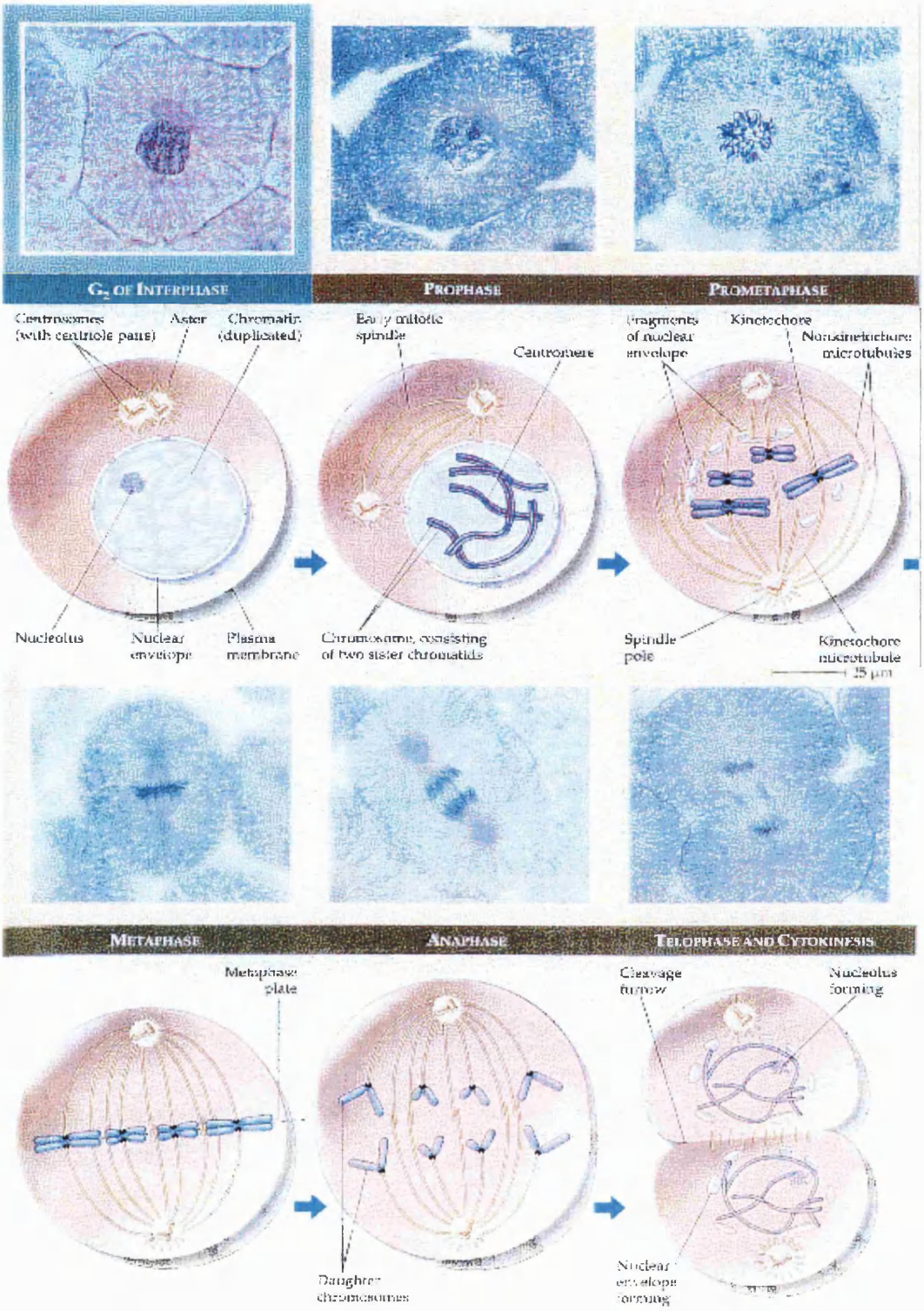


Figure 6.1 : Stage of mitosis[110]

M phase includes both mitosis and cytokinesis, is usually the shortest part of cell cycle. Successive mitotic cell divisions alternate with a much longer interphase, which often accounts for about 90% of the cell cycle. It is during interphase that the cells grows and copies its chromosomes in preparation of cell division. During G1, S and G2 phases, the cell grows by producing proteins and cytoplasmic organelles. Thus the cell grows (G1), continues to grow as it copies its chromosomes (S), grows more as it completes preparations for cell division (G2), and divides (M). The daughter cells formed may then repeat the cycle. Typically, a cell completes its cycle in about 24 h, but some cells like those in human liver, have a cycle lasting more than a year. Many non-dividing cells (all quiescent fibroblasts) in tissues suspend the cycle after mitosis and just prior to DNA synthesis. Such resting cells are said to have exited from the cell cycle and to be in the G0 state.[109] At any one time most of the cells in an animal's body are in G0 phase. Some, such as muscle and nerve cells, remain there permanently and others such as liver cells can resume G1 phase in response to factors released during injury.

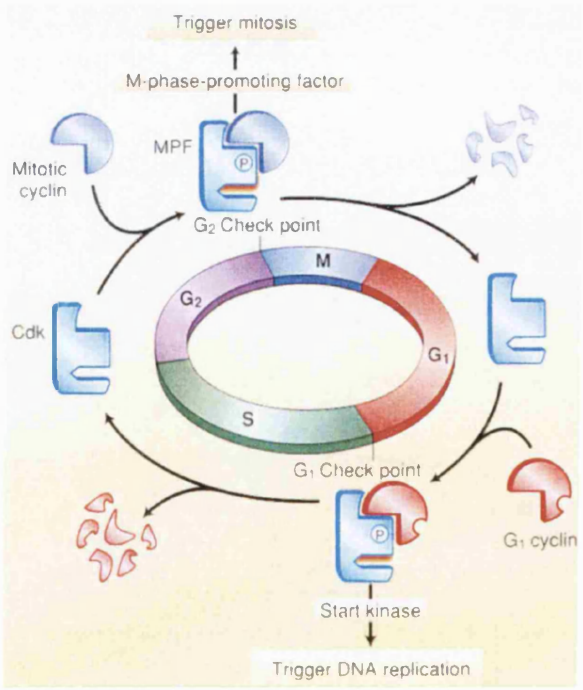


Figure 6.2 : Different phases of interphase[110]

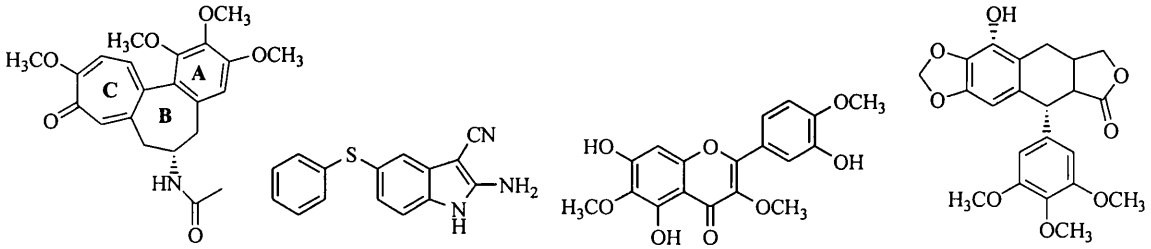
Microtubules, therefore are intimately involved with the replication of cells. If the microtubules in a tumour cell can be prevented from forming or decaying, the chromosomes cannot separate, the cell cannot reproduce and hence the tumour cannot grow. Thus agents, which interfere with the dynamics of tubulin may also act as inhibitors of cell division. Indeed, a number of these agents have shown to act as clinically useful anticancer agents.

6.7 Angiogenic, apoptotic indicing and antimicrotubular drugs

Three different small-molecule binding sites are known to date for the tubulin system. These are the colchicine site and the vinca alkaloid domain, both located on monomeric unpolymerised α,β-tubulin, and the taxoid site on the polymerised microtubule. Drugs, which bind to tubulin can be subdivided into separate classes.[311] The class into which a particular drug fits depends upon the effect, which that exerts on the binding of five well-characterised agents to tubulin. These agents are colchicine, the two vinca alkaloids, vincristine and vinblastine and the two macrocyclic natural products rhizoxin and maytansine. However, some drugs do not have any effects of the binding of these drugs, having affinity instead for a separate, distinct region of tubulin. Some of them may bind covalently to certain reactive groups on the protein, particularly the tubulin sulphydryl group. Ligands, which interact at the taxoid site stablise the microtubule, while ligands interacting at the vinca domain or the colchicine site disrupt the formation of microtubules. Treating the cells with these drugs can completely halt their replication.[311]

6.8 Inhibitors of tubulin-binding sites

Inhibitors of tubulin polymerisation interacting at the colchicine-binding site are potential anti-cancer agents. Colchicine **(6-1)** has long been associated with microtubules and it is indeed the classic tubulin binding agent[312] (Figure 6.3). Colchicine is a highly soluble alkaloid first isolated from the meadow saffron, *colchicum autumnale*.[313] It has been initially used for the treatment for gout but due to its high toxicity, is not commonly used.

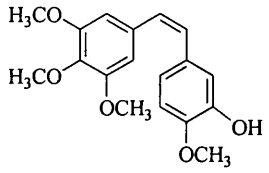


Colchicine **6-1** **Ampethinile** **6-2** **Centauriedin** **6-3** **Podophyllotoxin** **6-4**

Figure 6.3 : Inhibitors binding to the colchicine-binding site of tubulin

Colchicine induces a partial unfolding in the secondary structure of the protein caused by binding to a high affinity site on the tubulin, which hinders tubulin assembly,[314] It has been suggested that it is this unfolding which disrupts the protein regions necessary for microtubule formation.[315] Other drugs such as amphethinile **(6-2)**, a synthetic agent, which gained interest when it was shown to terminate pregnancy in rats[316] and later shown that it competitively binds to colchicine-binding site of tubulin effectively and hence entered Phase 1 clinical trial in the mid-80's.[317] The natural flavone centauriedin **(6-3)**, which is isolated from *Polymnia fruticosa* prevents the binding of colchicine to tubulin and prevents the formation of microtubules with an IC_{50} of 3 μM.[318] Although there are several other natural flavones have been isolated, centauriedin was the first flavone to cause mitotic arrest in cells stablising the mitotic spindle (Figure 6.3). For many hundreds of years, podophyllotoxin **(6-4)** was used to treat patients with conditions ranging from sclerosis of the liver through constipation, rheumatism and cancer. More recently, it has been shown to bind quickly and reversibly to at least part of the colchicine-binding site of tubulin.[319] This ease of reversibility and the low temperature dependence of binding, however, indicate that the binding does not occur at a completely identical site to that of colchicine. It is now thought that the predominant function is by inhibition of DNA topoisomerase II, an enzyme involved in the folding and unfolding of DNA during cell replication rather than by microtubular interactions.[320]

Combretastatin A-4 **(6-5)** is a naturally occurring stilbene first isolated in 1973 from the South African Bush Willow, *Combretum caffrum.*[321,322] It has been used by the Zulu as a charm to ward off enemies and in traditional medical practice (Figure 6.4). In 1979 some anti-cancer activity by this compound was found in the U.S. National Cancer Institute's astrocyte reversal (9ASK) system and the P388 lymphocytic leukimia cell line.[323] In 1982, Pettit and co-workers reported the structure of Combretastatin and later the synthesis.[321]



Combretastatin A
6-5

Figure 6.4: Structure of Combretastatin A

By far Combretastatin is one of the simpler and most potent inhibitors to show antimitotic effects by interacting with the colchicine-binding site of tubulin.[324] Combretastatin A-4 is not recognized by the multidrug resistance pump, a cellular pump, which rapidly rejects foreign molecules, including many anti-cancer drugs and it is also reported to inhibit angiogenesis.[325] Because of the low water solubility and poor bioavailability of this drug, it has limited *in vivo* efficacy, the progress into clinical trials was haltered.[326] But, recent studies have shown that in combination with radiation or chemotherapy it could form an effective therapy. Combretastatin A-4 is now being developed by the Cancer Research Campaign and the biotechnology company, Oxigene and believed to be available in the next five years.[327] This drug is effective against all solid tumours but the tumour must be at least 1 mm in diameter, about the size of a grain of rice and must have its own blood supply, since combretastatin acts by inhibiting the blood supply to the tumour cells and as a consequence the tumour cells are killed by lack of oxygen supply (Figure 6.5).

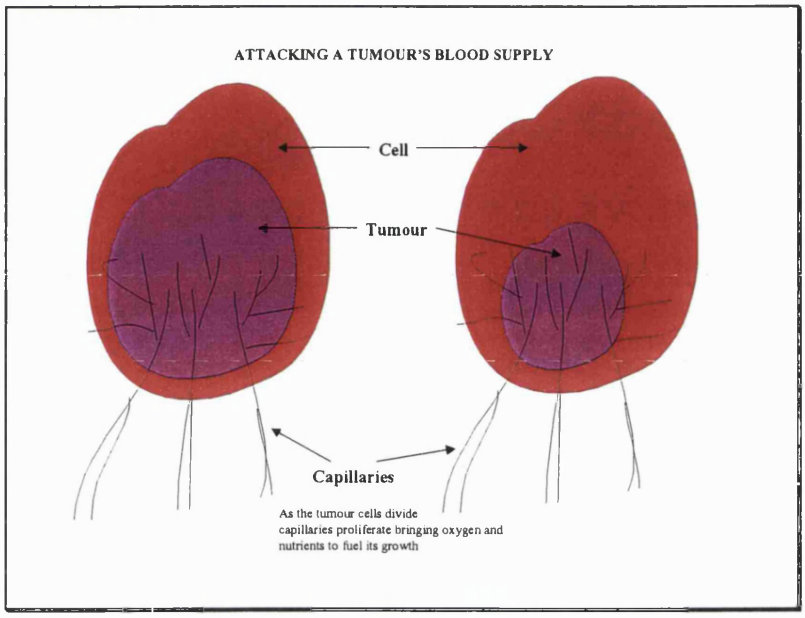


Figure 6.5 : Reduction of tumour size by drugs such as Combretastatin A-4, which attacks cells lining blood vessels to the tumour, cutting off the blood supply and causing the tumour to shrink.

Despite the vast number of colchicine-binding drugs available, several groups are now engaged in the synthesis of new potent combretastatin analogues with enhanced solubility, such as benzylaniline hydrochloride **(6-6)** (IC_{50} = 3.5 μM),[328] diphenylselenides **(6-7)**, diphenylsulphides **(6-8)**.[329] Combretastatin glucuronide **(6-9)** with attached sugar moieties,[330] and its phosphate prodrug CA4P **(6-10)**, which is water soluble and cause the disintegration of the vasculature of solid tumours and is currently in Phase I/II clinical trials (Figure 6.6). The new class of drugs Combretastatins of the D series **(6-11)** are now considered potential anticancer agents.[310]

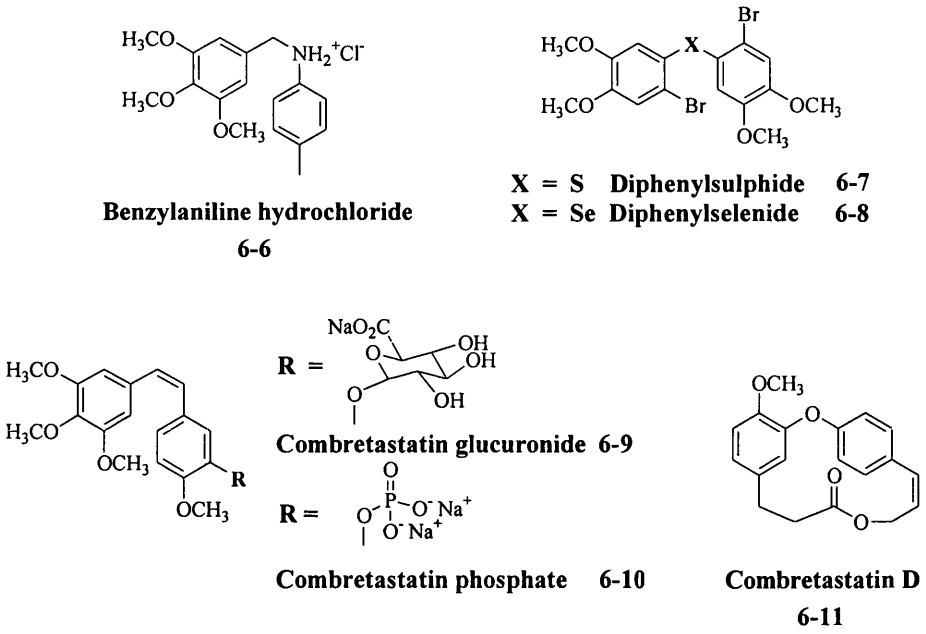


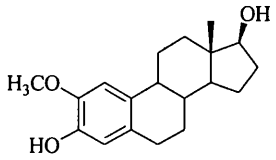
Figure 6.6 : Derivatives of Combretastatin A

6.9 2-Methoxyestradiol (2-MeOE2)

In 1957 Kraychy and Gallagher first reported the presence of 2-methoxyestrone in human urine after the administration of E2 or E1.[331] The naturally occurring metabolite of the mammalian hormone estradiol, 2-methoxyestradiol (2-MeOE2) **(6-12)** (Figure 6.7) is formed in the body by oxidation in the liver followed by *O*-methylation by catachol-*O*-methyltransferase (COMT), an enzyme present in large amounts in many organs and cells such as liver, kidney,[332] brain,[333] placenta, uterus, mammary glands and red blood cells (RBCs).[334,335].

2-MeOE2 is cytotoxic to several tumour cell lines, binds to colchicine-binding site of tubulin and induces the formation of abnormal microtubules. Interestingly, 2-MeOE2 does not appear to cause destabilisation of microtubules at concentrations sufficient for tubulin binding and mitotic block and cause little noticeable change in the morphology of assembled microtubules. It is thought that 2-MeOE2 exerts its effects by altering the dynamics of tubulin polymerisation. Many breast tumours initially show a response to commonly used anti-estrogens or aromatase inhibitors,

and most of them eventually become resistant to the therapy. 2-MeOE2, once considered an inactive end metabolite of E2 has recently emerged as a very promising agent for cancer treatment. It has shown to inhibit angiogenesis[336] and the growth of solid tumours. Knowledge of angiogenesis process provides opportunities for developing therapeutic agents such as 2-MeOE2, which can directly inhibit key stages of the angiogenesis cascade by selectively targeting intergral components such as the endothelial cells.



2-Methoxyestradiol

6-12

Figure 6.7 : Structure of 2-methoxyestradiol

Extensive studies have shown that methylated catachol estrogens have little or no binding affinities for the classical ER (<0.1% compared with E2), and lack estrogenic activity in the uterus.[337] Hence this raises the question of why the body makes such a large amount of this hormonally inactive, lipophilic estrogen metabolite. Studies have led to the suggestion that metabolic *O*-methylation may be a rapid inactivation or detoxification process for those estrogen derived catechols and mainly that 2MeOE2 is not just an inactive metabolite of E2 but, it has a unique protective role against estrogen induced carcinogenesis in estrogen target organs.[338,339,340,336]

Decreased formation of 2-MeOE2 in the body increases the risk of estrogen-induced cancers.[332] However, the growth inhibitory and pro-apoptotic mechanism(s) of action of 2-MeOE2 compounds is not fully resolved. Several mechanisms have been suggested to explain the antiproliferative activity of 2-MeOE2 such as: tubulin binding, up-regulation of p53[341] and deregulation of cell

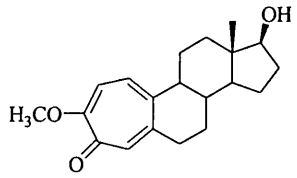
cycle kinases and regulators, such as $p34^{cdc2}$ and cyclin B.[342] 2-MeOE2 has been shown to induce changes in the levels and activities of various proteins involved in the progression of the cell cycle and these include, cofactors of DNA replication and repair, inhibition of mitosis, uneven chromosome distribution, faulty spindle formation and an increase in the number of abnormal metaphases.[340,339] Their is evidence to indicate that these effects are the results of inhibition of tubulin polymerisation by 2-MeOE2 to the colchicine binding site.[336] 2-MeOE2 binds relatively weakly to the colchicine binding site on tubulin, and inhibits the polymerisation of tubulin dimers to give microtubules.

Other possible mechanisms that have been suggested are those involving transcription factor modulators, (SAPK/JNK)[343] the regulators of cell arrest and apoptosis (tubulin,[336,344] p21, bcl-2 and FAS), proliferating cell nuclear antigen (PCNA),[345] nitric oxide synthase[346] and stress activated protein kinase.[347] 2-MeOE2 arrests cells in mitosis and induction of apoptosis is associated with phosphorylation of the anti-apoptotic bcl-2 protein. However, there is no evidence for any of these being part of a common mechanism operating in different systems. Recently, it has been found that 2-MeOE2 upregulates the KILLER/Death Receptor 5 (DR5) and activates the downstream caspases, caspase 8 and caspase 3 in endothelial and tumour cells. The antiproliferative activity of 2-MeOE2 is thought to be associated with the induction of apoptosis, which is independent of the estrogen receptor.[348]

Whatever the structural identity of the putative intracellular effector or receptor for 2-MeOE2, the interaction of 2-MeOE2 with its specific effector or receptor in target cells is an initial step leading to the expression of its growth-inhibitory effects in sensitive cells. Hence an understanding of the mechanism of action of 2-MeOE2 should further enhance our understanding of its unique physiological and its antitumourigenic functions.

6.10 Derivatives of 2-MeOE2

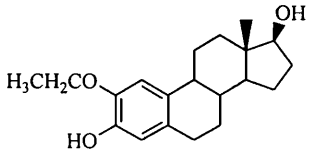
To further enhance the activity of 2-MeOE2, several groups extensively studied the Structure-Activity of A, B and C ring modified analogues of 2MeOE2 and some were more potent inhibitors of tubulin polymerisation and/or cytotoxic agents than the parent compound.[348,349,350,351] D'Amato *et al* proposed that A-ring of 2MeOE2 is functionally equivalent to the C ring of colchicine and that the CD rings of the steroid correspond to the trimethoxybenzene A ring of colchicine.[336] On the basis of this hypothesis, several A ring expanded analogue of 2MeOE2 (estratropones) **(6-13)** were synthesised by Miller *et al* and demonstrated to be more potent inhibitors of tubulin polymerisation than 2-MeOE2 itself[352,353] (Figure 6.8).



Estratropone
6-13

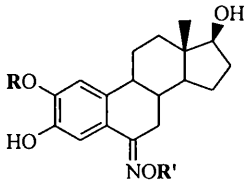
Figure 6.8 : A ring expanded analoges of 2-MeOE2

In an effort to investigate the structural parameters associated with the biological activities of 2-MeOE2, Cushman and his colleagues synthesised some structurally similar compounds with different substituents at the C-2 position of the 2-MeOE2. Most potent of these new analogues was proved to be 2-ethoxyestradiol **(6-14)**, which was found to be more potent than 2MeOE2 as a cytotoxic agent in cancer cell cultures as well as tubulin polymerisation inhibitor.[350] They also identified several 2EtOE2 analogues such as 2-EtO-6-oximinoE2 **(6-15)**, 2-(2',2',2'-trifluoroethoxy)-6-oximinoE2 **(6-16)** and 2-EtO-6-methoxyiminoE2 **(6-17)** to be potent inhibitors of tubulin polymerisation[351] (Figure 6.9). This observation of variability of the C-6 substituents suggests that the A ring of 2MeOE2 interacts with the same portion of the tubulin molecule as the C ring of colchicine and the C-6 substituents are probably analogous to C-7 side chain in colchicinoids.



2-Ethoxyestradiol

6-14



R = Et	**R'** = OH		**6-15**
CH_2CF_3	OH		**6-16**
Et	OCH_3		**6-17**

Figure 6.9 : Analogues of 2-ethoxyestradiol

6.11 Taxol

The most well known of these anti-tubulin agents are taxol (paclitaxel) **(6-18)** and taxotere (docetaxel) **(6-19)**, which are currently used in the clinic effectively (Figure 6.10). Taxol was first isolated from *Taxus brevifolia* bark extracts in 1962 as part of the American National Cancer Institute (ANCI) natural product screen of over 35,000 plant extracts in 22 years. Taxol was like every antitubulin compound known at that time was considered a microtubule stabilizer rather than a destabilizer, but it was largely ignored due to low availability and poor water solubility. Over the years the level of interest in this compound has increased, resulting in four groups have successfully completed the total synthesis and now one of the most popular areas of pharmaceutical interests. Taxol is now clinically used to treat a wide spectrum of malignancies including lung, head, neck, Kaposi sarcoma,[354] ovarian and advanced breast cancers together with ceramide-enhancing agents (ceramide is a known messenger of apoptosis) to maximise its cytotoxic potential.[355] When taxol was combined with doxorubicin, 90% response rate was observed, but shown to be cardiotoxic, which was reduced when doxorubicin was replaced with epirubicin.[356] Also various other combination of drugs with taxol such as mitoxantrone/taxol[357] are also shown to be effective.

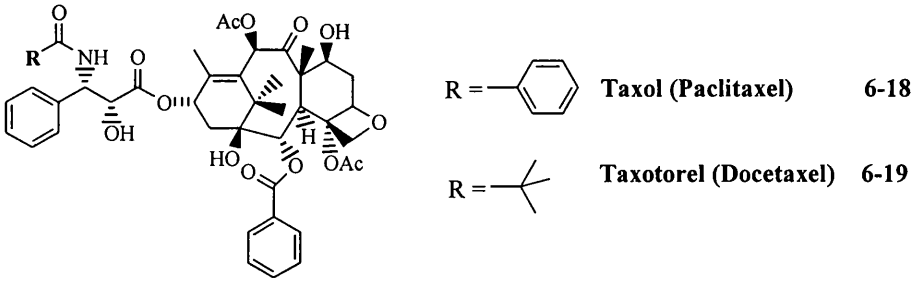


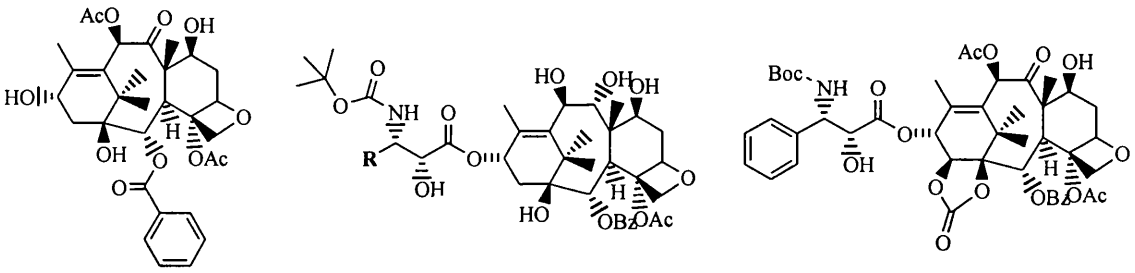
Figure 6.10 : Structures of Taxol and Taxotorel

The effect of taxol on microtubules is well established. Taxol induces tubulin to assemble into microtubules under normally prohibitive conditions and stabilises these polymers against disassembly. It binds to the single site of the β-tubulin and suppresses the dynamic behaviour of microtubules i.e. the cell proliferation in a concentration-dependent manner.[358] Incubation of cells with taxol leads to the formation of abnormal bundles of microtubules and results in the arrest of cells in the G2/M phase of the cell cycle.[359]

The discovery of several other natural or synthetic analogues of taxol, which structurally deviate from the taxoid platform and superficially do not resemble each other, has proven of great significance in the field. Baccatin III **(6-20)** is one such compound isolated from the needles of yew and a synthetic side chain was attached to the baccatin core.[358] and its analogue ABT-271 **(6-21)**, which posses an α-hydroxyl group at C-9 instead of the carbonyl in Taxol or taxotere. These compounds are identified to be promising anti cancer agents with activities similar or better than Taxol[360] (Figure 6.11).

The discovery of the second receptor for taxol[361] has added further layer of complexity to the understanding of the structural requirements for cytotoxic activity, since little is known about the interaction of the taxol analogues with the receptor. *Bcl-2* family proteins play a crucial role in the regulation of apoptosis. Recent findings show that taxol inducd apoptosis and *Bcl-2* protein phosphorylation

when treated with human hepatocellular carcinoma cell line, QGY-7703.[362]



Baccatin III
6-20

R = iPr **ABT-271** **6-21**
R = CF_2H **6-22**

IND 5109
6-23

Figure 6.11 : Analogues of Taxotorel

Taxotere was discovered as a late-stage intermediate in a synthesis of taxol and now it has been obtained semi-synthetically from a precursor readily obtained from the needles of the yew tree. Taxotere shows similar spectrum of action to taxol however, it reduces the complexity and side effects of administration in comparison to taxol because it is four-fold more potent than taxol and shows improved water solubility.[363] It has now been licensed for the treatment of ovarian, advanced breast cancer and non-small cell lung cancers. The fluorinated CF_2 analogues **(6-22)** synthesised by Ojima *et al* showed higher potency against drug sensitive cell line LCC6-WT and 40-70 times potent against drug resistant cell line LCC6-MDR and induce apoptosis as compared to that of taxol.[364] Another analogue, IDN 5109[365] **(6-23)** is active on multidrug resistant cells and found to be easier to administer, orally active and endowed with better profile of side effects.[363]

Therefore it can be clearly seen than the concept of inhibiting a number of key biochemical processes, in particular the cell division and cell growth hold considerable potential for future therapeutic development.[366] Angiogenesis and apoptosis are some of these processes in cancer that can be exploited in controlling tumour cell proliferation. However, the differences in such processes can be small as a result of alterations or damage to only a minute proportion of normal cellular

processes and hence the design and synthesis of a selective anticancer drug to target the individual cellular processes specifically has yet to be accomplished.

CHAPTER 7

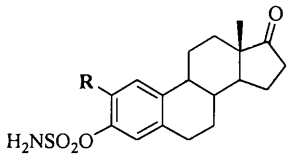
CHAPTER 7

7.0 Aims of this work

It can be very clearly seen from the different types of drugs that are reported, that 2-methoxyestrogens are an important class of new drugs that can be used to inhibit tumour growth and angiogenesis. Although the identification of 2-methoxyestradiol (2-MeOE2) **(6-12)** (Figure 6.7, Chapter 6) as a new therapy for cancer is a remarkable advance, the bioavailability of orally administered estrogens is generally poor and also they can undergo extensive metabolism when they pass through the liver. This problem could be overcome by designing a prodrug of the active agent, which would reach the target effectively before it can be cleaved off to release the drug molecule to elicit the desired effect. From previous investigations it has been identified that EMATE **(2-20)** (Figure 2.9, Chapter 2), a synthetic drug, which was initially synthesised as a steroid sulphatase inhibitor for breast cancer therapy, is also a super estrogen. Its oral uterotrophic activity in rats has been found to be about 100-times greater than that of E2.[197] Its enhanced estrogenicity is thought to be the result of the drug being sequestered into the red blood cells after absorption and hence protected from inactivation during its passage through the liver and possibly act as a reservoir for its subsequent slow release from the erythrocytes for a prolonged period of time.[266] Although the mechanism for the desulphamoylation of EMATE remains to be established, it can be envisaged that EMATE is acting as a prodrug of E1, with its sulphamate group cleaving off *in vivo* to release E1 possibly after its inactivation of steroid sulphatase.

With the super-estrogenicity of EMATE being a disadvantage, a number of A-ring modified analogues of EMATE were synthesised and tested. Among these, 2-methoxyestrone-3-*O*-sulphamate (2-MeOEMATE) **(2-40)** was found to be equipotent to EMATE as a steroid sulphatase inhibitor but devoid of estrogenicity[206,367] (Figure 7.1). In addition to all the compounds that are known to possess antimitotic activity, 2-MeOEMATE has demonstrated some superior qualities that lacked in others. From the DNA analysis it was apparent that 2-MeOEMATE like 2-MeOE2 and taxol, induces

G2/M arrest of the cell cycle. In an experiment carried out by Purohit and co-workers it was shown that cells that are treated with 2-MeOEMATE remained arrested in the G2/M phase for at least 24 h, even after washing off the drug.[206] When a similar experiment was carried out with 2-MeOE2, a significant proportion of the cells went back to G1/S phase after the removal of the drug[368] providing further evidence that 2-MeOEMATE is possessing potent and prolonged antiproliferative effects.[206]



R = OCH_3 **2-MethoxyEMATE 2-40**
CH_2CH_3 **2-EthylEMATE 2-42**

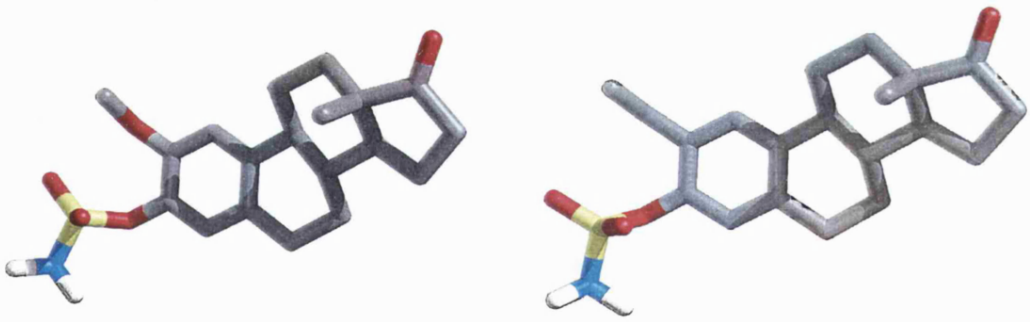
Figure 7.1 : 2-Substituted analogues of EMATE

The inhibition of the growth of MCF-7 cells as a result of the induction of apoptosis by 2-MeOEMATE was shown by Purohit *et al.*[208] In untreated cells, only a few rounded and detached cells were visible per field whereas, for cells treated with 2-MeOEMATE (1 μM) for 24 h there was a significant increase in the number of both rounded and detached cells. As the stromal compartment constitutes a major proportion of the volume of breast tumours, the effect of 2-MeOEMATE on the morphology of breast tumour-derived fibroblasts was examined by Purohit *et al.*[208] At 1 μM, little effect on cell morphology was detected, whereas at 5 μM rounding of a significant proportion of fibroblasts was observed. In contrast to its effects on epithelial cells, 2-MeOEMATE did not reduce the number of fibroblasts. After an exposure to 2-MeOEMATE, a significant increase of 27% of the total cells was in the sub-G1/S phase of the cell cycle fraction, and represented apoptosis.[369] This is likely due to the increase in the phosphorylation of the Bcl-2 oncoprotein (antiapoptotic).[343] Phosphorylation of Bcl-2 occurs during the arrest of cells in the G2/M phase of the cell cycle[370] and blocks its ability to dimerise with Bax (proapoptotic protein) and hence causing the induction of apoptosis.

2-MeOEMATE, unlike other compounds is found to be active against both ER^+ and ER^- breast tumours and also a number of other types of tumour cell lines. A marked

effect on the growth and the morphology of ER^+ MCF-7 cells and ER- MDA-MB-231 breast cancer cells was observed with 2-MeOEMATE.[206] The effect of the sulphamoyloxy group in 2-MeOEMATE is studied and it was found that 2-MeOEMATE inhibited MCF-7 cells proliferation by 52% at 1 μM, whereas the precursor 2-MeOE1 had only a little effect.[371] This clearly indicates that the potency observed for 2-MeOEMATE was due to the presence of the sulphamoyloxy group, which may be acting as a prodrug of 2-MeOE1 or may be exhibiting enhanced binding to the colchicine-binding site of tubulin.

In vivo, 2-MeOEMATE also inhibited the growth of some nitrosomethylurea-induced mammary tumours in intact rats.[369] The tumour regression due to 2-MeOEMATE was compared with that of 2-MeOE1 and found that the tumours receiving 2-MeOEMATE regressed almost completely, whereas 2-MeOE1 receiving tumours only showed a modest regression, indicating that the sulphamate is much more potent than its parent compound. In addition, 2-MeOEMATE has been recognized to be non-toxic as the body weight of the animals were found to be unchanged.

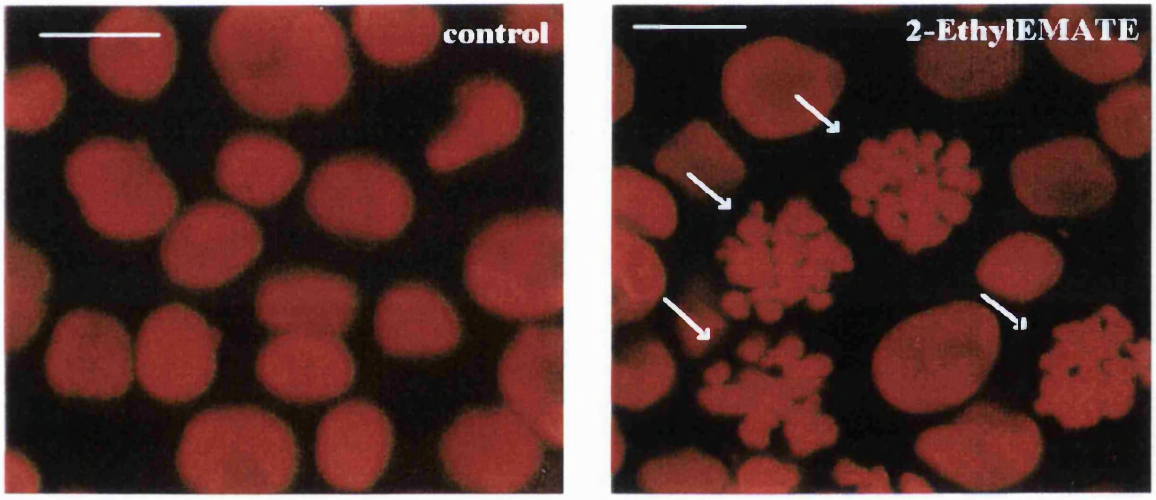


2-MeOEMATE **2-EthylEMATE**

Figure 7.2 : Pictorial representation of 2-MeOEMATE **(2-40)** and 2-ethylEMATE **(2-42)** by Sybyl®

Cancer cells do not have the ability to store glucose therefore they depend upon constant uptake to sustain growth. Glucose enters cells *via* Na^+/glucose co-transporters such as GLUT 1-7.[372] Transfection of cells with oncogenes *ras* or *src* increases the rate

of glucose uptake and hence the expression of glucose transporter mRNA.[373] Inhibiting the glucose uptake and metabolism by cancer cells is another attractive new therapeutic target. A number of compounds such as the isoflavone genistein and cytocholasin B have been shown to inhibit the process of glucose uptake[374] as well as disruption of cell actin cytoskeleton by disrupting the actions of the glucose transporters.[375] MCF-7 cells have a high capacity to take up a glucose analogue, deoxyglucose. 2-MeOEMATE has been shown to possess the ability to inhibit this insulin-stimulated uptake and hence inhibit cell proliferation better than the non-sulphamoylated parent compound.[376] It is anticipated that alterations in microtubule stability interfere with the recruitment of glucose transporters to the cell membrane.[375]



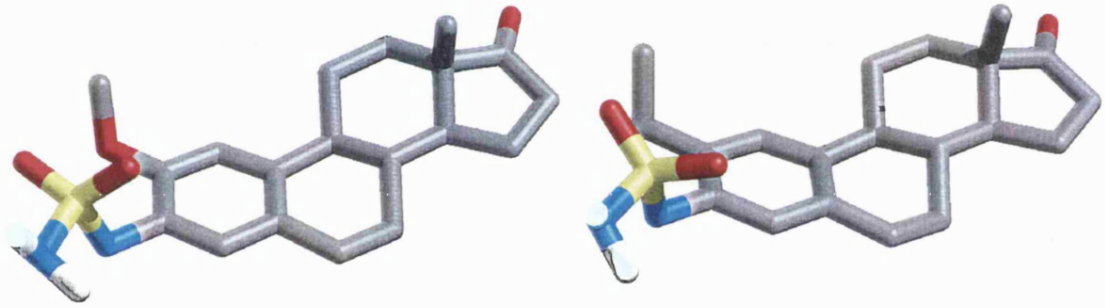
(A) **(B)**

Figure 7.3 : Apoptosis caused by 2-ethylEMATE. **(A)** Control **(B)** cells treated with 2-ethylEMATE.

Like 2-MeOEMATE, another 2-substituted derivative 2-EtEMATE **(2-42)** (Figure 7.2) was also found to promote the inhibition of tubulin polymerisation and induce apoptosis. (Unpublished) Apoptosis caused by 2-ethylEMATE is shown in figure 7.3. The advantages of 2-MeOEMATE and 2-EtEMATE over other compounds are that they are orally bioavailable and it is possible that these sulphamates are acting as prodrugs of their parent compounds. Like EMATE, it is possible that they also may be

taken up by red blood cells and released slowly to give a protracted increase in blood steroid concentration and hence a reduction in cell proliferation. This concept is still to be validated but if this is the case, relatively high levels of drug could be delivered over a prolonged period of time to attack tumour growth and these drugs therefore hold considerable potential for therapeutic development.[366]

In view of the exciting results obtained for the sulphamates, it is indeed interesting to establish some degree of structure-activity relationship for the 2-MeOEMATE class of compounds by synthesising derivatives with surrogates of the sulphamate group. The obvious modification is to replace the bridging O atom in the sulphamoyloxy group (H_2NSO_2O-) with other heteroatoms. Therefore 2-substituted estrone-3-sulphamide (Figure 7.4) and 3-*S*-sulphamates were synthesised in this project.

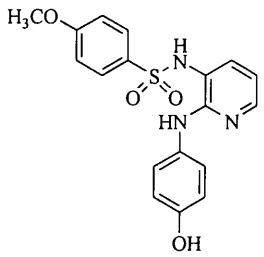


2-MeOestrone-3-sulphamide **2-Ethylestrone-3-sulphamide**

Figure 7.4 : Pictorial representation of 2-MeOestrone-3-sulphamide and 2-ethylestrone-3-sulphamide by Sybyl®

These surrogates are resistant to hydrolysis than the sulphamate group. Hence, their biological activities would help to establish if the increase in potency observed for 2-MeOEMATE over 2-MeOE1 is the result of sulphamoylation of essential amino acid residues at the receptor-binding site. If these surrogates shared similar biological activities to 2-MeO or 2-ethylEMATEs, it is possible that the enhanced potency observed for 2-MeOEMATE could well be due to the result of better binding affinity than 2-MeOE1 to the receptor possibly through hydrogen bonding provided by its sulphamate group.

It has been recently reported that a series of sulphamido antimitotic agents of which compound E7010 **(7-1)** (Figure 7.5) has been shown to inhibit microtubule formation by binding to the colchicine-binding site.[377] *In vivo*, tests have shown good oral activity across a wide range of tumour types and good activity against vinca alkaloid resistant solid tumours. Results from animal studies carried on E7010 have indicated that this agent is active against gastric, colorectal, breast and lung cancer tissues. The clinical viability of this drug is currently being investigated in Phase I clinical trials.[378]



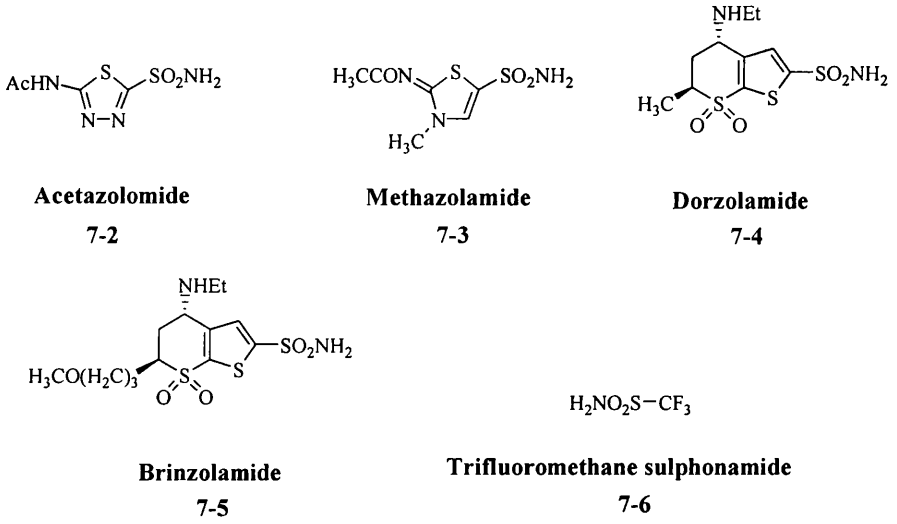
E 7010
7-1

Figure 7.5 : Structure of E7010

E7010 contains a sulphamido group although its not clear if such a moiety is crucial for its biological activities. Since the sulphonamide and the sulphamoyloxy groups are similar in structure, an obvious target modification to 2MeOEMATE and 2-EtEMATE is to synthesise their 3-sulphamido derivatives.

In addition, these compounds chosen to synthesise would further provide an indication about the importance or need of the sulphamoyloxy group for the potent activity observed in the sulphamates. Certain sulphonamides such as acetazolamide **(7-2)**, methazolomide **(7-3)**, dorzolomide **(7-4)** and brinzolomide **(7-5)** are known inhibitors of carbonic anhydrase, which catalyses the interconversion of CO_2 and HCO_3^- [379] (Figure 7.6). Their clinical use has been in the reduction of the formation of aqueous humour for the treatment of glaucoma, which is a chronic ophthalmic condition affecting approximately 15 million people.[379] The binding mechanism of

trifluromethane sulphonamide inhibitor **(7-6)** (Figure 7.6) to carbonic anhydrase II is well established by the help of X-ray crystallography. It has been shown that the N-atom of the sulphonamide tetrahedrally coordinates directly to the active site zinc ion of the enzyme, a small hydrophobic moiety placed in the hydrophobic part of the active site and the hydrogen bonds provided by the oxygens are the factors making **7-6** high affinity for carbonic anhydrase.[380]



Acetazolomide
7-2

Methazolamide
7-3

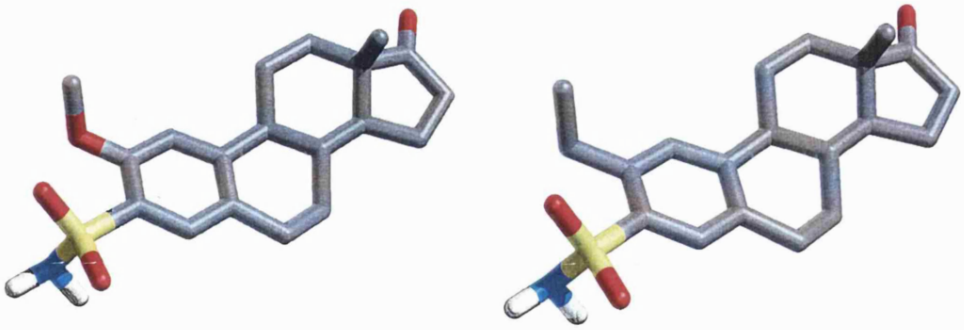
Dorzolamide
7-4

Brinzolamide
7-5

Trifluoromethane sulphonamide
7-6

Figure 7.6 : Structures of some known carbonic anhydrase inhibitors.

It has been hypothesised that EMATE's super-estrogenicity might be the result of it being sequestered into erythrocytes, hence evading 'first-pass' metabolism.[371] It is possible that the same mechanism is also operable for 2-MeOEMATE rendering the compound more effective than 2MeOE1 in inducing tumour regression *in vivo* as well as possibly causing enhanced binding to the colchicine site. Since the sulphonamido and sulphamoyloxy groups are similar in structure and that there is a high level of carbonic anhydrase in red blood cells, it is possible that the sequestration of EMATE and related compounds such as 2-MeOEMATE into red blood cells operates in the same manner as that for sulphonamides. In order to assess this hypothesis, the 3-sulphonamido derivatives of 2-substituted estrones were prepared (Figure 7.7).



2-MeOestrone-3-sulphonamide **2-Ethylestrone-3-sulphonamide**

Figure 7.7 : Pictorial representation of 2-MeOestrone-3-sulphonamide and 2-ethylestrone-3-sulphonamide by Sybyl®

It is reasonable to expect that the 2-substituted sulphonamide derivatives would also show similar inhibitory activities to that of the sulphamates as a result of being taken up into the blood cells possibly with minimal metabolism. Most importantly, these compounds would offer useful information about the nature of the site where the sulphamoyl group binds in tubulin. This site might be one of the five binding sites that have been previously identified such as colchicine site or might be a new sulphamate-binding site unique to the sulphamoyl group, which is possible but unlikely. It might also indicate how the sulphamoyloxy group binds to tubulin to produce the irreversible inhibition as observed, which might be similar to that of sulphamoylation of sulphatase enzyme. Therefore several important aspects of inhibition by sulphamoylated compounds are likely to be revealed as a result of this study.

7.1 Synthesis

The 2-substituted estrogen derivatives synthesised in this project can be represented by the general structures as shown below in Figure 7.8: 2-Substituted estrone **(A)** sulphamides **(B)** sulphonamides and **(C)** *S*-sulphamates.

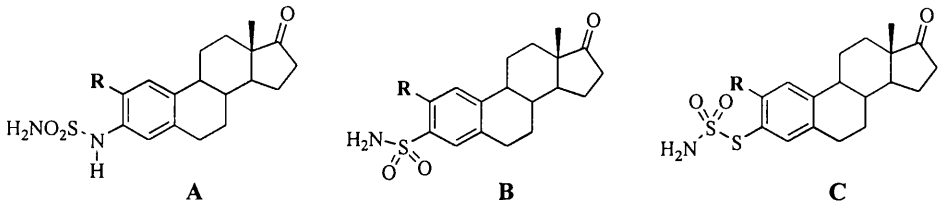


Figure 7.8 : General structures of the 2-substituted derivatives synthesised in this project. R = H, OCH_3 or Et.

In view of the established promising biological activity with 2-MeOEMATE and 2-MeOE2MATE, multi-gram quantities of 2-MeOE1 were required in order to prepare the corresponding sulphamides and sulphonamides. An efficient synthetic method for the preparation of 2-MeOE1 was required, which could provide the target compounds rapidly in large quantities. The ideal method should also provide flexibility for incorporating other alkyl or alkoxy groups to the C-2 position without too much difficulty.

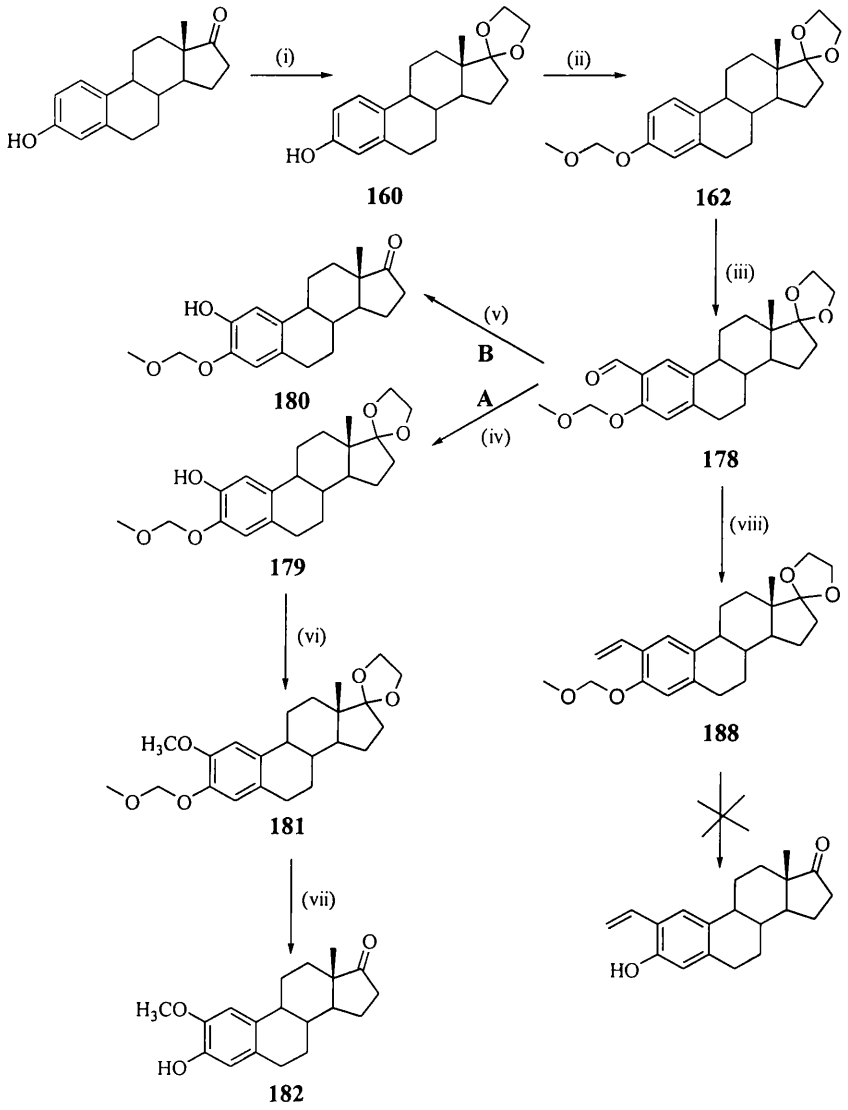
A literature search has revealed several different approaches to the synthesis of 2MeO estrogens but most lacked selectivity and gave low yields. A practical synthesis was first reported by Fishman *et al*[381] starting by condensation of estriol with 2-chloro-5-nitrobenzophenone. This method has been proved to be cumbersome. Since the publication of Fishman's method, many synthetic procedures have been reported. Rao *et al* demonstrated a two step synthesis[382] *via* the formation of 2-bromoestradiol from estradiol itself. This procedure however requires long reaction time of 22 h and the yield is not satisfactory. Numazawa *et al*'s approach to obtain 2-MeO estrogens from 2-iodo estrone supposedly furnished a good yield (65%) and gave good regioselectivity.[383] The four-step method by Nambara *et al* was reported to be a viable one for the synthesis of 2-methoxy estrogens, although it was found to be inefficient in practice.[384]

Direct hydroxylation at the C-2 position is another approach, which could also be employed to synthesise 2MeO estrogens. Taylor *et al* have reported the use of thallium

(III) trifluoroacetate (TTFA), which is an efficient and simple method for phenol preparation.[385,386] Many other methods focused on the introduction of an acyl or formyl group at the C-2 position[350,384,387] followed by an oxidation with *m*-chloroperoxybenzoic acid (*m*CPBA) to give the 2-hydroxyl, which can eventually be alkylated. To carry out this approach the 3-hydroxyl group must be protected with a suitable protecting group, which would not be cleaved under acidic conditions such as that of Baeyer-Villiger oxidation.

One probable approach is to carry out *ortho*-lithiation selectively at the C-2 position of C-3 hydroxy- and C-17 carbonyl- protected estrone. Pert *et al* have shown that *ortho*-lithiation can be selectively carried out at the C-2 position by protecting the C-3 hydroxyl group with a MOM group (CH_3OCH_2-).[443] A similar procedure was effectively utilised by Cushman *et al*, in their multi-step synthesis of 2-alkyloxyestradiols where they used benzyl ether as the protecting group of the 3- and 17-hydroxyl, which was removed with palladium (10% or black)/palladium hydroxide on charcoal in ethanol/THF mixture.[350,351] However this method is lengthy and too many by-products are produced due to cleavage of the benzyl protecting groups at various stages of the whole synthesis. Therefore, to overcome some of the disadvantages of Cushman's method, a modified procedure developed in Bath by Leese *et al*[388] was employed, where estrone instead of estradiol has been used as the starting material with its C-17 carbonyl protected with ethylene ketal (Scheme 7.1).

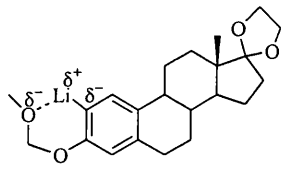
A mixture of estrone, ethylene glycol ($HOCH_2CH_2OH$) and a catalytic amount of *p*-toluenesulphonic acid (*p*TsOH) in toluene was refluxed under boiling using Dean-Stark conditions to gave the C-17 acetal- protected compound **160** in 96% crude yield (Scheme 7.1). Several similar methods have been reported previously for the protection of the C-17 carbonyl group of steroids with an acetal group.[389] Cyclic ketals are stable to bases and basic nucleophiles and formed by the reaction of two molecules of alcohol with one molecule of ketone. Ethylene glycol is a single molecule with two terminal hydroxyl groups (diol) and has been effectively used for protecting the C-17 carbonyl. Cyclic ketals are more resistant to acid hydrolysis than acyclic ones and are also easier to synthesise.



Scheme 7.1 : Synthesis of 2-methoxyestrone **(182)**. (i) $HOCH_2CH_2OH$, toluene, *p*TsOH, Δ, 14 h (ii) NaH, 0°C, CH_3OCH_2Cl, DMF, R.T., 12 h, N_2 (iii) THF, –78°C, N_2, ^{sec}BuLi, DMF, 12 h (iv) *m*CPBA, CH_2Cl_2, $NaHPO_4$, N_2, 3 h, R.T., NaOH/MeOH, HCl (v) *m*CPBA, $CHCl_3$: CH_2Cl_2 = 1:3, 24 h, R.T. (vi) DMF, CH_3I, tetra Bu_4NI, N_2 (vii) CH_3OH, 0°C, N_2, acetyl chloride (viii) THF, $KOBu^t$, $MePPh_3I$, R.T., 2h.

Methyl chloromethyl ether (MOMCl - CH_3OCH_2Cl), which was synthesised from a mixture of dimethoxymethane ($CH_3OCH_2OCH_3$), decanoyl chloride and concentrated sulphuric acid and was used for the subsequent protection of C-3 hydroxyl group. The phenolic ion formed by the deprotonation of C-3 hydroxyl with NaH, reacts with MOMCl to give the acetal, which is stable to bases and can be subsequently cleaved easily by mild acid treatment. The pure product **162** (Scheme 7.1) was obtained as a thick clear syrup in 95% yield, which solidified on standing.

Upon treatment with ^{sec}butyl lithium, compound **162** was selectively *ortho*-lithiated at the C-2 position.[443] MOM group in **162** effectively directs the lithiation at the C-2 position, a finding exploited by Cushman *et al* in their synthesis of 2-alkoxyestradiol.[350,351] The flexibility of this synthetic approach to 2-substituted estrones is illustrated further when alternative electrophiles are used to quench the lithiated estrone intermediate **A** (Figure 7.9). The use of freshly distilled DMF afforded the aldehyde **178** in an excellent yield of 98% (Scheme 7.1).



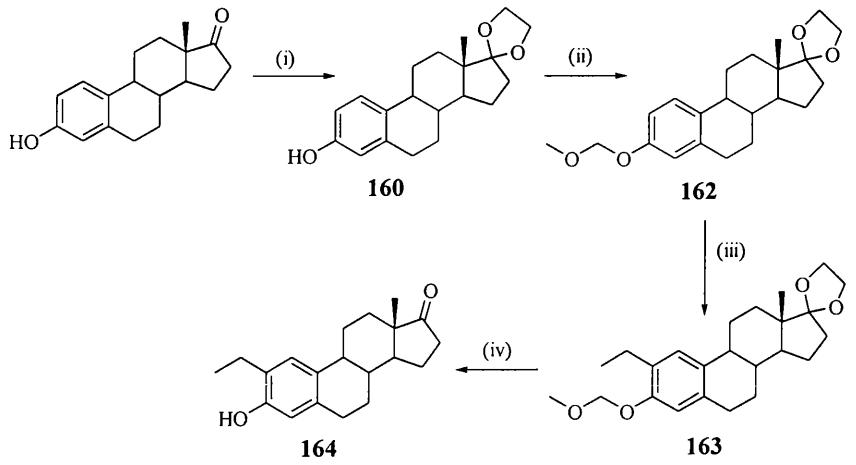
A

Figure 7.9 : O*rtho*-lithiation of compound **162**.

The aldehyde **178** formed was oxidised using Baeyer-Villiger oxidation by two different methods. One approach was where *m*CPBA was used in the presence of $NaHPO_4$ buffer in CH_2Cl_2. The subsequent acid hydrolysis of the resulting formate, yielded the phenol **179** in good yield (80%) (Scheme 7.1, Path A). Similar to Path A, the purified *m*CPBA was added to a solution of **178** in a 1:3 mixture of $CHCl_3$: CH_2Cl_2 at R.T. to give compound **180** in 61% yield (Scheme 7.1, Path B). The ratio of the two solvents employed seemed to be a key factor for this reaction since the slightly acidic nature of this mixture of solvents seems to be important. Although the yield is relatively low and the reaction time is longer, method B gave a cleaner reaction than

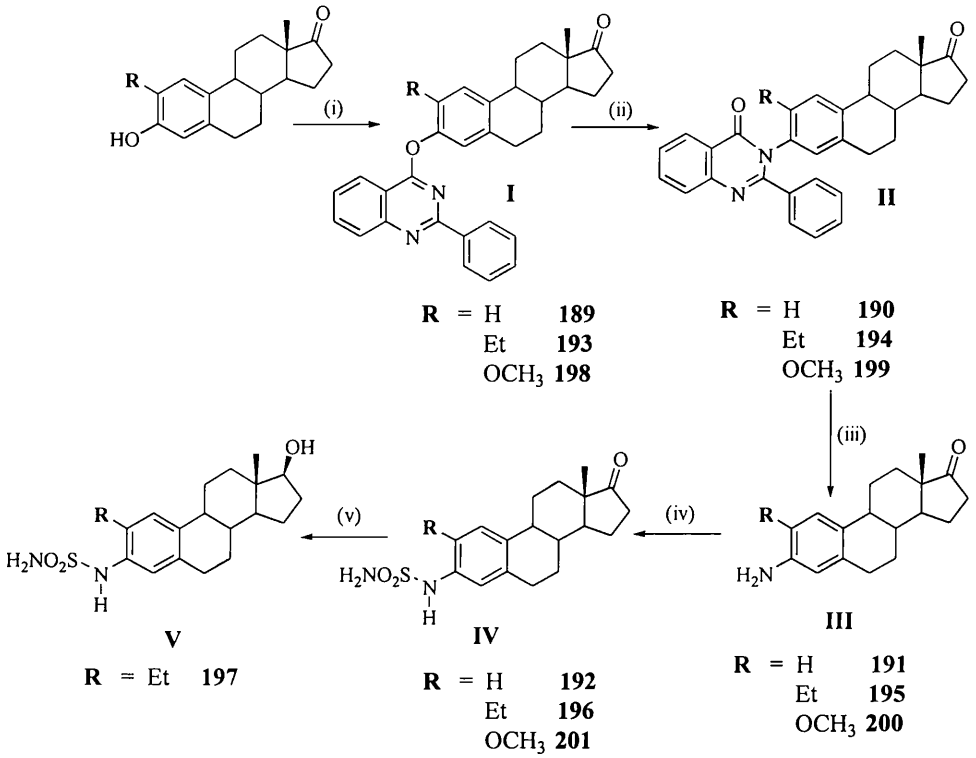
method A with an easier purification process. However, the proton NMR spectrum of the product obtained revealed the absence of the C-17 ketal group. This is presumably due the presence of acidic impurities in the mixture of chlorinated solvents or the *p*-chlorobenzoic acid, which formed after the oxidation of *m*CPBA might be responsible for the cleavage of the C-17 ketal group. Therefore, method B is not suitable for any reactions that may require C-17 carbonyl protection as a ketal. In this particular sequence of reactions, the presence of this C-17 ketal group is not vital for the subsequent reactions. Therefore, method B can also be employed to syntheise the 2-hydroxylated compound. Compound **179** in DMF was then alkylated with methyl iodide using anhydrous K_2CO_3 as base to give compound **181** in 90% yield. The C-3 and C-17 protecting groups in compound **181** were subsequently cleaved with HCl, which was generated *in situ* from acetyl chloride in methanol to give the desired 2-methoxyestrone **182** in an excellent yield of 70% (Scheme 7.1).

In an attempt to convert the aldehyde **178** into a vinyl group at the C-2 position, a Wittig reaction was employed. The Wittig reaction is extremely useful and a versatile method for the synthesis of alkenes from aldehydes. The aldehyde **178** was added to a THF solution of methyltriphenylphosphonium iodide containing potassium ^{tert}butoxide base at R.T. The ylide, formed by the abstraction of a proton from the methyl group of the phosphonium salt by the base, acting as a carbanion attacks the aldehyde carbon of the C-2 formyl group to give an oxophosphatane intermediate, which decomposes to give the required alkene **188**. Compound **188** however failed to give the desired 2-alkenyl-3-hydroxyestrone upon acidification, where HCl was generated *in situ* from acetyl chloride in methanol. A mixture of products was obtained, which proved difficult to isolate into individual components (Scheme 7.1).



Scheme 7.2 : Synthesis of 2-ethylestrone **(164)** (i) $HOCH_2CH_2OH$, toluene, TsOH, Δ, 14 h (ii) NaH, 0°C, CH_3OCH_2Cl, DMF, R.T.,12 h, N_2 (iii) THF, –78°C, ^{sec}BuLi, EtI, R.T., 12 h, N_2 (iv) CH_3OH, 0°C, N_2, acetyl chloride.

This highly flexible synthetic approach to 2-substituted estrones by Leese *et al*[388] gave way to the synthesis of 2-alkylated derivatives of estrone using the method shown in Scheme 7.2. Different electrophiles can be employed to alkylate the 2-lithiated derivative. In this case, iodoethane was used to obtain the C-3 and C-17 protected 2-ethylestrone **163** in 91% yield. This intermediate was subsequently acid hydrolysed to re-generate the desired 2-ethylestrone **164** in an excellent yield of 95%. The method reported by Cushman *et al* also provides an effective way to synthesise 2-alkylestradiols where they have prepared a series of 2-alkylated estradiols by reaction of 2-formylestradiol with a series of Wittig reagents.[350]



Scheme 7.3 : Synthesis of 2-substituted estronesulphamides (i) diglyme, NaH, N_2, 4-chloro-2-phenylquinazoline, Δ, 3 h (ii) mineral oil, N_2, Δ (iii) ethanol, $NaOH/H_2O$, Δ, 7 h, N_2, HCl (iv) DMF, NaH, N_2, 0°C, H_2NSO_2Cl, 0°C to R.T. (v) isopropanol /THF, $NaBH_4$, 30 min., 0°C.

Once the parent 2-MeOE1 and 2-EtE1 were made in adequate quantities, they were utilised for the subsequent reactions to make the desired sulphamides and sulphonamides. Several other methods have been described in the literature for the synthesis of 3-amino derivatives starting from phenols and estrone. Most were by the conversion of simple phenols to anilines, since phenols generally are more widely available than the corresponding anilines. One of the possible reactions is the Bucherer reaction, which works well with naphthalenes and related heterocycles, whereas benzene derivatives are much less reactive.[390] Smiles rearrangement is another useful method reported and could be carried out in one pot.[391] Unlike these procedures, Gold and Schwenk postulated a method by the condensation of steroidal quinol acetate and

benzylamine. However, the resulting product proved to be difficult to dissolve and purify.[392]

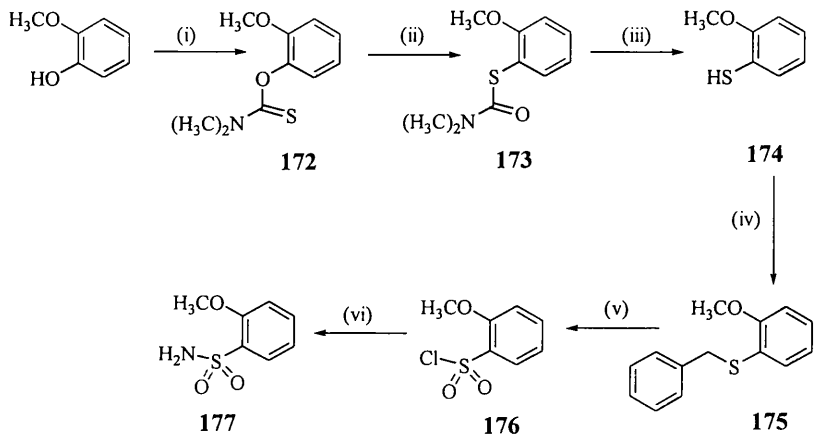
The method described by Morrow and Hoffer for the synthesis of 3-aminoestrone was adopted here for the synthesis of 2-substituted estrone sulphamides.[393] Even though the reactions involve rearrangement of atoms at extremely high temperatures and require basic conditions, this method seems to be more practical than the others and is expected to provide a relatively higher yield. The sodium salts of E1 derivatives were condensed with 4-chloro-2-phenylquinazoline to give the corresponding 2-substituted 3-(2'-phenyl-4'-quinazolinyloxy)estrone **(I)** (Scheme 7.3). As well as using 4-chloro-2-phenylquinazoline, estrone can also be activated by diethyl chlorophosphate to give a similar product to that of **I** (aryl diethylphosphate esters).[394] But it is not desirable since diethyl chlorophosphate is toxic and also involves the usage of potassium metal in liquid ammonia. Bis(2-methoxyethyl) ether (Diglyme) was used as the solvent, which is water soluble and removed easily during the aqueous work-up procedure. The corresponding products were obtained as crystalline solids in excellent yields (**189**, **193** and **198** = 80%, 70% and 83%, respectively) (Scheme 7.3).

The derivatives of **I** were thermally rearranged to yield the corresponding 3-[4'-oxo-2'-phenyl-3'(4H)-quinazolinyl]estrone **(II)**. For this thermal rearrangement, very high temperatures were attained by melting the compounds in heavy mineral oil (bp = > 400°C). The rearrangement temperatures varied from compound to compound. For the unsubstituted estrone derivative **(190)** the temperature required was found to be between 300–310°C and for the 2-ethyl **(194)** and 2-methoxy **(199)** derivatives the temperatures required were higher at 320–330°C and 310–320°C respectively (Scheme 7.3). These temperatures were found to be crucial for the rearrangements to take place. No reaction took place at a few degrees below the optimum temperature range whereas charring and decomposition took place at few degrees above these specific temperature ranges. It took several attempts before the correct temperatures for the rearrangement of an ether to an amide was optimised. Another practical problem encountered with this reaction was that the 2-methoxy derivative of **II** gave a suspension in mineral oil

and was soluble in all the common organic solvents used to remove the mineral oil such as hexane and petroleum spirit. Therefore, the product was extracted into acetone and purified by flash chromatography or recrystallisation with hot isopropanol to obtain the products in good yields (**190**, **194** and **199** = 79%, 67% and 60% respectively). The relatively lower yield observed for the 2-methoxy derivatives was due partly to the cleavage of the 2-methoxy group at high temperature to give compound **190**. This fraction has been isolated and identified by proton NMR analysis, and found to be corresponding to the E1 derivative of **II**. i.e. **190**. The relatively poor yield of **199** could also be due to decomposition of compound and the production of a few unisolatable byproducts.

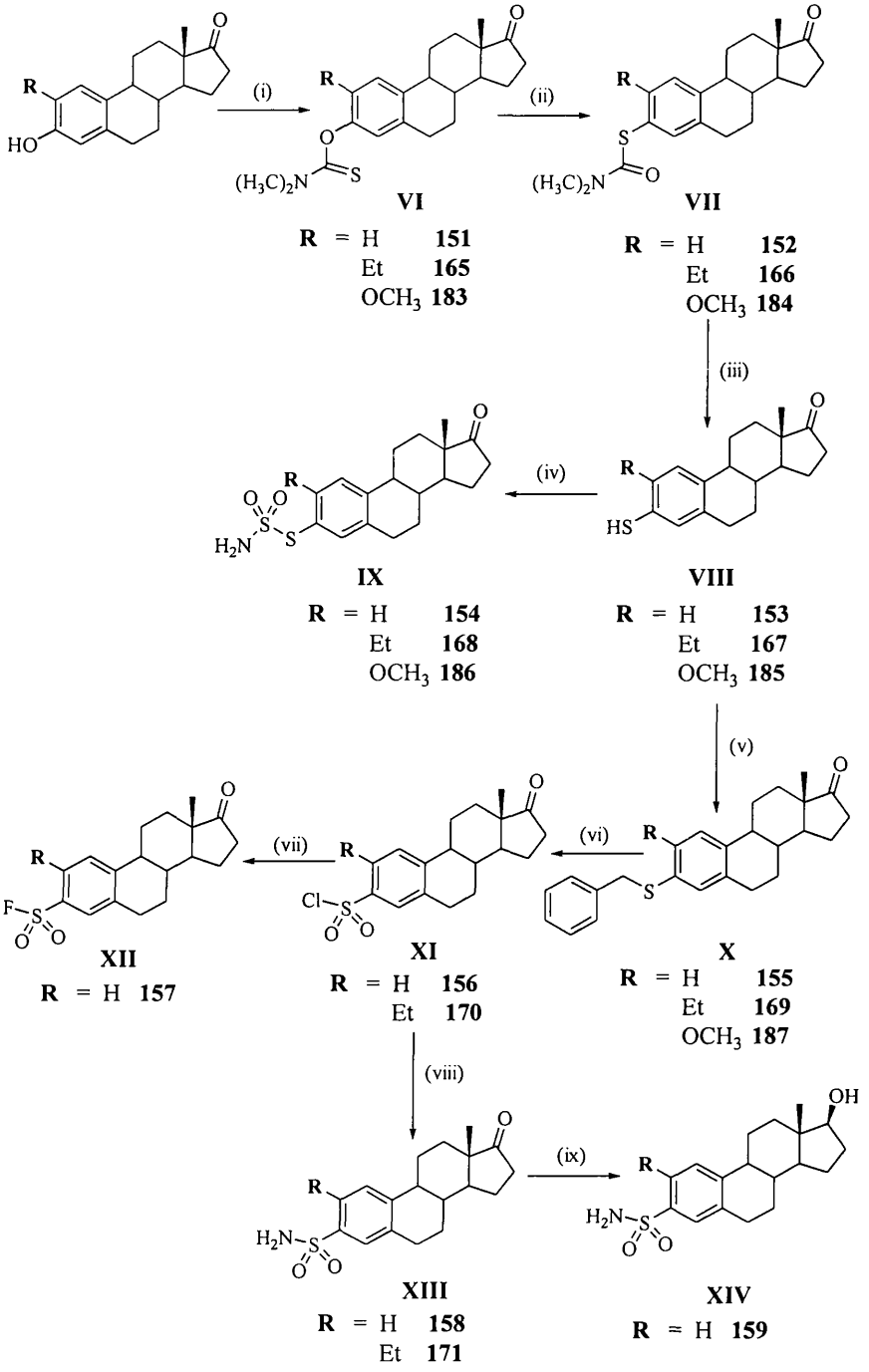
3-Aminoestrone **(III)** and its 2-substituted derivatives were obtained upon basic hydrolysis of **II**. The product **III** was purified either by flash chromatography or direct recrystallisation from hot isopropanol [**191** (16%), **195** (36%) and **200** (66%)]. (Scheme 7.3). In contrast to the literature method,[393] in which 3-aminoestone was purified by sublimation followed by recrystallisation with a benzene-cyclohexane mixture. Treating **III** with sulphamoyl chloride by the usual method yielded the corresponding 2-substituted sulphamides **(IV)**. The yield of sulphamides obtained was rather poor [**192** (17%), **196** (29%) and **201** (14%)] (Scheme 7.3). It was observed that the sulphamoylation reaction did not go to completion as anticipated even with a large excess of sulphamoyl chloride (>5 eq.) as confirmed by the retrieval of the unreacted **III** in a significant amount.

The natural metabolite 2MeO estradiol and the synthetic agent 2MeOE2MATE were proven to be highly potent antitubulin agents.[369] Therefore, the C-17 carbonyl group of compound **196** was reduced stereoselectively to a 17β-OH group with $NaBH_4$ to give compound **197** as white crystals (Scheme 7.3). The C-17 proton was observed as a triplet at δ 3.73 ppm with a J value of 8.6 Hz. Even though the OH signal was not visible it was confirmed by the presence of the broad peak present at 3446 cm^{-1} in the corresponding IR spectrum. The sharp C=O stretch present in the spectrum of compound **196** at 1725 cm^{-1} had also disappeared.



Scheme 7.4 : Synthesis of 2-methoxybenzenesulphonamide **(177)**. (i) NaH/DMF, N_2, dimethylthiocarbamyl chloride, Δ, 80°C, 1 h (ii) mineral oil, Δ, N_2 (iii) NaOH, $EtOH/H_2O$, Δ, N_2, 4 h (iv) NaH/DMF, N_2, BnBr, R.T., 4 h (v) $AcOH/H_2O$, Cl_2, 15 min. (vi) NH_4OH, acetone, R.T., 15 min.

For the preparation of 2-substituted sulphonamides, the modified method described by Li *et al* was employed.[184] This procedure was similar to that of the synthetic route designed by Holt *et al* for the synthesis of 5α-reductase inhibitors.[395] This method was previously demonstrated by Woo *et al* in their synthesis of unsubstituted estrone-*S*-sulphamate[194] and also by Newman *et al* in their synthesis of various arylthiocarbamates.[396] A similar procedure was used to synthesise 2-methoxy benzenesulphonamide, (Scheme 7.4) which was made as a model for the target compound, 2-methoxyestronesulphonamide (Scheme 7.5). The desired 2-methoxybenzenesulphonamide **(177)** was obtained in an excellent yield of 80%. The same method was employed for the synthesis of 2-methoxy and 2-ethylsulphonamide by treating the corresponding estrone derivatives with NaH and *N,N*-dimethylthiocarbamoyl chloride in DMF to form the corresponding *O*-aryl thiocarbamates **(VI)** in good yields [**151** (80%), **165** (106% crude) and **183** (74%)] (Scheme 7.5).



Scheme 7.5 : Synthesis of estrone sulphonamide **(158)** and its 2-substituted derivatives. (i) NaH/DMF, N_2. dimethylthiocarbamoyl chloride, Δ, 80 °C,1 h (ii) mineral oil, Δ, N_2 (iii) NaOH, EtOH/H_2O, Δ, N_2, 4 h (iv) NaH/DMF, N_2, 0°C, H_2NSO_2Cl, 0°C to R.T. (v) NaH/DMF, N_2, BnBr, R.T., 4 h (vi) AcOH/H_2O, Cl_2, 15 min. (vii) 1,4-Dioxane, KF/ H_2O, Δ 50 - 55°C, 24 h (viii) NH_4OH, acetone, R.T., 15 min. (ix) isopropanol/THF, $NaBH_4$, 30 min., 0°C.

The *O*-aryl thiocarbamates were then thermally isomerised to the corresponding *S*-aryl thiocarbamates **(VII)** through Newman-Kwart rearrangement by heating in heavy mineral oil[396] (Figure 7.9). The temperatures for the rearrangement of atoms were rather high and varied for each compound. For the unsubstituted estrone derivative **(152)** the temperature required was between 280–290°C whereas for the 2-ethyl **(166),** 2-methoxy **(184)** and 2-methoxybenzene **(173),** derivatives the temperatures were found to be 250-260°C, 270°C and 280°C, respectively. The yields of the 2-methoxy derivatives **173** and **184** were found to be relatively poor (48% and 45% respectively). It is possible that the extremely high temperature employed for the rearrangement reaction cleaved the 2-methoxy moiety resulting in a reduction of the isolated yield of the products **173** and **184** compared to the unsubstituted and 2-ethyl derivatives [**152** (76%) and **166** (92%)] (Scheme 7.5). The mechanism of the rearrangement reaction presumably involves a nucleophilic attack of the sulphur atom on the carbon at which the oxygen atom is attached as depicted in Figure 7.10. The dialkylamino group of the carbamate assists the desired polarisation.

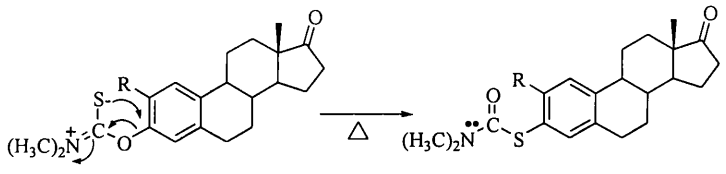


Figure 7.10 : Proposed mechanism of the thermal rearrangement of *O*-arylthiocarbamates to *S*-arylthiocarbamates

In the subsequent reaction, the thiophenol **(VIII)** was obtained by alkaline hydrolysis of **VII** with NaOH in aqueous ethanol in good yields except for the 2-methoxy derivative **185**, which again gave a rather low yield of 25% [**153** (86%), **174** (74%), **167** (89%)] (Scheme 7.5). There are not many practically applicable methods available for the synthesis of thiophenols. Although the yield of thiophenols *via* the formation of thiocarbamate is not very impressive, this is a fairly reasonable method to follow in the laboratory with ease.

Sulphamoylation of **VIII** with an excess of sulphamoyl chloride by the usual method gave the corresponding *S*-sulphamate **(IX)** in low yields of the desired products [**154** (56%), **168** (28%) and **186** (14%)] (Scheme 7.5). Once again, it was observed that the reaction does not go to completion and hence most of the starting materials were recovered. These compounds were identified by ^{1}H NMR analysis and difficulties were encountered in obtaining good mass spectroscopy data since the *S*-sulphamates are very unstable for the conditions used for FAB^+ and E.I. Very soft techniques such as electrospray (e.s.) were required to detect their molecular ions. Stability of these compounds is an important factor since it determines whether these compounds are indeed stable in the bioassay conditions. Chemical stability studies carried out in assay conditions by Woo *et al*[194] for the unsubstituted estrone derivative of **IX**, compound **154** and also for compound **192** (Scheme 7.3) on HPLC showed that these sulphamates do not degrade to form the corresponding parent steroid, indicating most likely these compounds are also stable under the biological assay conditions used to test these compounds.

The thiophenol derivatives **(VIII)** were deprotonated with NaH in DMF, followed by alkylation of the resulting thiolate anion, which acts as a nucleophile in an S_N2 reaction, with benzyl bromide to obtain the corresponding thiobenzyl derivatives **(X)** in good yields except for the 2-methoxy derivative **187** which gave the lowest yield of 21% [**155** (82%), **175** (88%) and **169** (65%)]. The sulphonyl chloride derivatives **(XI)** were obtained by oxidation with chlorine in acetic acid-water [**156** (78%), **176** (94%) and **170** (55%)]. As well as the desired sulphonyl chloride, such oxidation is expected to furnish the benzyl acetate and benzyl chloride as by-products (Figure 7.11).

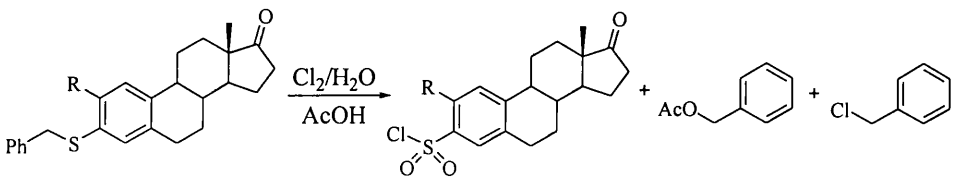


Figure 7.11 : Reaction of thiobenzyl derivatives with Cl_2

Unfortunately, when this reaction was carried out with the 2-methoxy derivatives, the desired product was not formed. The reaction cleaved off the 2-methoxy group and also a lot of inseparable byproducts were obtained. This is presumably due to the formation of the cation **(B)**, which undergoes further reactions with the surrounding anionic species to produce a mixture of compounds (Figure 7.12) or the 2-methoxy group could be cleaved as depicted in figure 7.13, to form the stable product **C**. Disappointingly, repeating the reaction at various different conditions such as lower temperatures, a reduction of the reaction time with Cl_2 or the use of highly diluted reaction mixture did not yield the desired product. Although the same reaction worked well before for the transformation of **175** to **176**, (Scheme 7.4) somehow it proved to be difficult with the 2-methoxyestrone derivatives. This observed difference in reactivity with the estrone derivative may be due to the formation of chloride radicals during light reaction, attack at the benzylic positions (Shown with * in Figure 7.12) of the estrone molecule and hence producing a mixture of products. Therefore, conducting the experiment in the dark could provide a solution to the difficulty encountered in the process. Direct oxidation of the thiophenol **185** did not give the product either. The synthetic utility of benzylic sulphides as the intermediates in the synthesis of sulphonyl chloride is superior to that of from thiophenol since a benzylic group is selectively cleaved rather than a potentially complex steroid molecule.

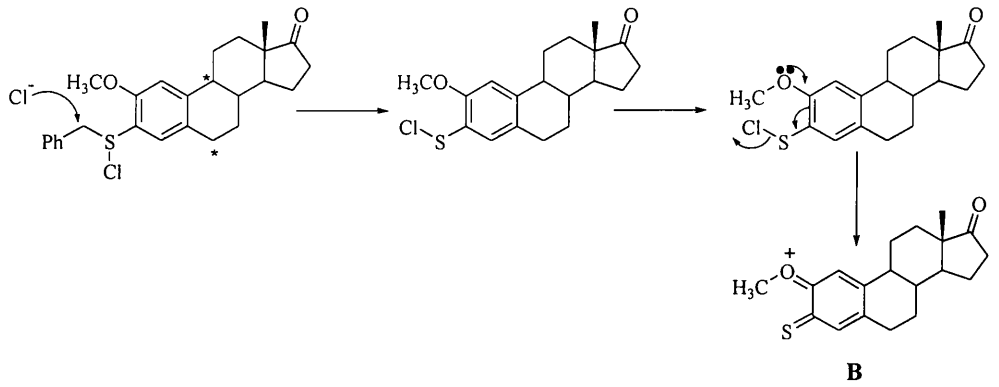
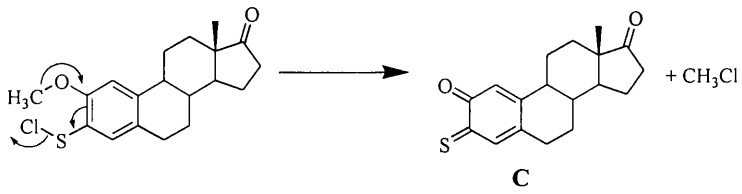


Figure 7.12 : Reaction of Cl_2 with the 2-methoxy containing compound

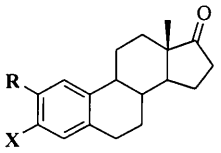


C

Figure 7.13 : Possible demethylation mechanism of the 2-methoxy derivative

The sulphonyl fluoride derivative **(XII)** was obtained in 60% yield with the unsubstituted estrogen derivative **157** by halogen exchange reaction using potassium fluoride. (Scheme 7.5) Treatment of **XI** with ammonium hydroxide yielded the desired sulphonamides **(XIII)** in excellent yields [**158** (82%), **177** (80%) and **171** (61%)]. The estradiol derivative of unsubstituted estrone-3-sulphonamide **159** was prepared in 26% yield by reduction of **158** with excess $NaBH_4$ in THF/isopropanaol (1:1) mixture (Scheme 7.5). The C-17 proton appeared as a triplet at δ 3.53 ppm with J = 8.6 Hz. The C-17 OH group peak was observed as a singlet at δ 4.54 ppm, which was exchanged with D_2O.

The following key compounds were synthesised during the course of this project:



	R	X		R	X		R	X
154	H	SSO_2NH_2	**168**	CH_2CH_3	SSO_2NH_2	**186**	OCH_3	SSO_2NH_2
158	H	SO_2NH_2	**171**	CH_2CH_3	SO_2NH_2	**201**	OCH_3	$NHSO_2NH_2$
192	H	$NHSO_2NH_2$	**196**	CH_2CH_3	$NHSO_2NH_2$			

Table 7.1 : Compounds synthesised in the sulphamides, sulphonamide and *S*-sulphamate series

In addition, the estradiol derivatives, estra-1,3,5(10)-trien-17-ol-3-sulphanamide **(159)**, 2-ethylestra-1,3,5(10)-trien-17-ol-3-sulphamide **(197)** and 2-methoxy benzylsulphonamide derivatives, DGS01142B **(177)** were also synthesised.

7.2 Results

In this work, several A-ring modified novel 2-substituted estrone-3-sulphamides, 3-sulphonamides and 3-*S*-sulphamates derivatives were designed and synthesised. The studies on the ability of these compounds to inhibit E1-STS activity and to cause tubulin polymeristion, angiogenesis and apoptosis are currently underway. Therefore, the biological activity data for these compounds are not available at present.

7.3 Conclusion

The vast amount of natural and synthetic drugs that are available as potential microtubule disrupters highlights the importance of tubulin as a target for anticancer agents and the structural diversity in compounds that interact at various sites of tubulin. Any compound in this category may be suitable for the treatment of cancers such as breast, ovarian, endometrial, pancreatic, prostate cancers, sarcomas, melanomas and other solid tumours.

The identification of 2-methoxyestradiol (2-MeOE2) **(6-12)** (Figure 6.5, Chapter 6) as an antitumourigenic and antiangiogenic agent led to the synthesis of 2-methoxyestrone-3-*O*-sulphamate (2-MeOEMATE) **(2-40)** and related sulphamoyloxy analogues such as 2-methoxyestradiol-3-*O*-sulphamate (2-MeOE2MATE) **(2-50)** and 2-ethylEMATE **(2-42)** (Figure 6.10, Chapter 6). It has been well established that the sulphamoyloxy group is indispensable for potent irreversible inhibition of STS activity. 2-MeOEMATE was found to induce G2/M cell cycle arrest and modest levels of apoptosis in breast cancer cells *in vitro,* and also induced tumour regression *in vivo* in intact rats whereas, the parent compound 2-MeOE1 failed.[366]

The role of 2-MeOEMATE in the observed enhancement of the antiproliferative activity of 2-MeOE2 and its derivatives remains unclear. This raised questions as to

whether the sulphamoyloxy group may be the key structural factor for the observed activity. The rationale for this work is to find solutions to several unaswered questions resulted from the previous findings. It has been shown that 2-MeOE2 has a very low affinity for ER, and its activity comes from the binding to the colchicine binding site of tubulin resulting in either inhibition of tubulin polymerisation or formation of a polymer with altered stability, properties and morphology depending on the reaction conditions. Therefore it is interesting to investigate the exact mechanism and the site of binding of 2-MeOE2 to tubulin. The observation shows that 2-MeOEMATE, 2-MeOE2MATE and 2-ethylEMATE are more potent than 2-MeOE2 itself and they also might be acting in a similar manner indicating that the sulphamate group may be involved in an increased binding to tubulin. The irreversible inhibition of the tubulin polymerisation observed in the sulphamoylated compounds might be the result of sulphamoylation of tubulin in a similar way to that of sulphatase.

EMATE is a prodrug of E1, in which the sulphamate group is cleaved off *in vivo* to elicit effect at the target. Similarly, it is possible that 2-MeOEMATE and 2-MeOE2MATE may be acting as prodrugs of 2-MeOE1 and 2-MeOE2 respectively. It is also important to find out the binding mechanism of 2-MeOE2 and 2-MeOEMATE to the tubulin site. Therefore, the ultimate objective of this work became the modification of the sulphamoyloxy group, in order to establish some degree of structure-activity relationships for the 2-MeOEMATE class of compounds. Hence, a series of stable sulphamate surrogates such as 2-substituted estrone-3-sulphamides, 3-sulphonamides and 3-*S*-sulphamates were synthesised in this project as potential microtubule disruptors and antiangiogenesis agents.

The biological evaluation of these 2-substituted analogues is in progress at present. These results might lead to a greater understanding of the binding mechanism of these compounds to tubulin. It would also enable further studies to design and maximise the activity of metabolically stable and potent E1-STS inhibitors devoid of estrogenicity, since it is known that 2-MeOE2 and 2-MeOEMATE are non-estrogenic.[269] Unfortunately, the nature of these of inhibition of these compounds can only be

validated once the biological activity results are made available, which is currently going ahead.

CHAPTER 8

CHAPTER 8

Experimental

8.0 Chemicals and analyses

All reagents and solvents used were of general purpose or analytical grade unless otherwise stated and were obtained commercially either from Aldrich Chemicals, Lancaster synthesis or Maybridge.

Analysis by thin layer chromatography (TLC) was carried out using commercially available aluminium plates (Merck Kieselgel 60 F_{254}, 20×20 cm, layer thickness 0.25 mm, Art. No. 5554). Products and starting material spots were visualised either under UV light at 254 nm (hydroxycoumarins are brightly fluorescent) or by using anisaldehyde solution (for β-keto esters) or by treating with a methanolic solution of phosphomolybic acid (PMA) followed by heating (for steroids) or by a combination of these methods. Preparative TLC was performed on pre-coated glass plates (Merk TLC Silica gel 60 F_{254}, 20×20 cm, layer thickness 2 mm, Art. No. 5717). Flash chromatography was performed using BDH Kieselgel 60, flash silica (SiO_2), of particle size, 70-230 μM as stationary phase and different solvents systems, such as chloroform, dichloromethane, acetone, methanol, ethyl acetate and hexane of varying ratios as the mobile phase.

Melting points of solid, crystalline compounds were determined using Reichert-Jung Kofler block melting point apparatus and were uncorrected. Boiling points of oils are reported in Torr. unless otherwise stated. Fast atom bombardment (FAB), electron ionisation (E.I.) or electrospray (e.s.) mass spectra and accurate mass spectra were recorded at the University of Bath Mass Spectrometry service on a VG 7070H Double Focusing Mass Spectrometer and VG Autospec instruments. *m*-Nitrobenzyl alcohol

(*m*-NBA) was used as the matrix for FAB experiments. For e.s. experiments, samples were prepared in 1:1 water/methanol with 1% acetic acid.

IR spectra were recorded on a Perkin-Elmer 782 FT-IR spectrometer, using KBr discs or Nujol mulls between NaCl plates. Elemental analysis was carried out at the University of Bath Microanalysis service.

The Proton (^1H) and DEPT-edited carbon (^{13}C) spectra were recorded as a solution in deuterated chloroform ($CDCl_3$) or methylsulfoxide (DMSO-d_6) with tetramethylsilane (TMS) as internal standard at the University of Bath NMR Service, using a Jeol EX 400 (400 MHz) NMR spectrometer. The chemical shifts (δ) are reported in ppm and the chemical shift of the proton of residual undeuterated $CHCl_3$ in $CDCl_3$ is ~7.27 ppm, acetone is ~2.05 ppm and those of DMSO is ~2.49 ppm. The peaks corresponding to OH and NH_2 are visualised by exchanging with D_2O.

The log P calculations, analytical and preparative high performance liquid chromatography (HPLC) are carried out on a Waters 600E analytical and semi Prep systems. A Waters Radialpak column (RP18, 8 × 100 mm) was used at a flow rate of 1 or 2 ml/min. Methanol and water was used at different proportions as the mobile phase. All the spectra were determined at wavelengths (λ_{max}) of 190 – 400 nm, using a Photo Diode Array (PDA) and visualised at 254 nm. The retention times (t_R) of the compounds were expressed in minutes. Full details of the log P calculations are given in the Appendix C.

X-ray Crystal structures of the tricyclic coumarin sulphamates were determined by Dr. M. F. Mahon at the Dept. of Chemistry of University of Bath. Full details are given in the Appendix D. Molecular modelling was carried out by Mr. K. Smith at the Department of Pharmacy and Pharmacology of University of Bath.

All reagents and solvents used were stored away from moisture and light and dried before use. All experiments were carried out at room temperature (R.T.), unless otherwise stated. Low temperature experiments were conducted in a Dewar flask containing either ice-water with NaCl for 0°C and carbon dioxide pellets with acetone for –78°C. Magnesium sulphate ($MgSO_4$) was used as the drying agent. The solvents used in the reactions were removed with a rotary evaporator at reduced pressure (water pump) and on stated occasions, under a high vacuum. Samples for analysis were dried beforehand in a drying pistol at low temperature (~40-50°C) *in vacuo*.

8.1 Preparation of sulphamoyl chloride

Sulphamoyl chloride was prepared according to the method described by Appel and Berger[253] and stored as a standard solution as described by Woo *et al.*[194] To chlorosulphonyl isocyanate (25.0 g; 177 mmol; 1.1 eq.) in freshly distilled, anhydrous and sulphur impurities-free toluene (150 ml) at 0°C and under N_2, freshly distilled formic acid (7.20 g; 156 mmol; 1 eq.) was added dropwise at such a rate so that the reaction did not become too violent. The resulting white gel was warmed gradually to R.T. and stirred overnight under N_2. Evaporation under high vacuum gave a pale yellow residue of crude sulphamoyl chloride (*ca.* 19–21 g). A standard solution was prepared by dissolving the crude in anhydrous, sulphur-free toluene and stored under a positive pressure of N_2, in the refrigerator. No titration was attempted on this sulphamoyl chloride solution whose molarity was (*ca.* 0.5–0.7 M) estimated according to the crude sulphamoyl chloride obtained after work up. An appropriate volume of this solution was freshly concentrated in *vacuo* before use.

Toluene and formic acid were purified according to the method previously described.[397] Toluene (1 litre) was cooled to 2-4°C and placed in a separating funnel. Any sulphur impurities present in the cold toluene were removed by washing with cold conc. H_2SO_4 (3×100 ml). Then washed with water (2×100 ml) followed by aqueous 5% NaOH and again with water until neutral, dried and stirred in sodium metal overnight

at R.T. and then fractionally distilled under N_2 (bp = 110°C) and stored in dark place under N_2. Formic acid, after being stirred overnight at R.T. with dried and finely powdered boron oxide was fractionally distilled under N_2 (bp = 100-101°C) and stored in a refrigerator under N_2.

8.2 Experimental Procedures

8.2.1 General methods for the synthesis of ethyl 3-oxo-alkanoates for the preparation of 4-alkylcoumarin sulphamates

Method A[236]

To ethyl potassium malonate (2.1 eq.) in MeCN (100 ml/5 g of acid chloride) at 10-15°C and under N_2 was added Et_3N (3.2 eq.), followed by $MgCl_2$ (2.5 eq.). The mixture was stirred at 20-25°C for 2.5 h and then at 0°C for 0.5 h before the corresponding acid chloride (1 eq.) was added dropwise during 25 min. The mixture was further treated with Et_3N (5 ml) and stirred overnight at 20°C. The evaporation residue was dissolved in toluene and re-concentrated. More toluene was added, stirred and cooled to 10-15°C before aq. HCl (1M, 50 ml) was added cautiously while keeping the temperature <25°C. The organic layer was washed with 1M aq. HCl (50 ml) and water. Drying, evaporation and distillation or chromatography ($CHCl_3$ or $CHCl_3$/acetone, 10:1) gave the corresponding ethyl alkanoylacetate.

Method B[239]

To anhydrous $SnCl_2$ (0.1 eq.) was added CH_2Cl_2 (~100 ml/5g of aldehyde), followed by ethyl diazoacetate (1.05 eq.). The reaction was initiated by adding a few drops of the corresponding aldehyde in CH_2Cl_2. When N_2 evolution began, the remaining solution of aldehyde (1 eq.) was added dropwise over 30 min. After the evolution of N_2 had stopped (~1-3 h), the mixture was washed with brine (50 ml) and extracted twice

(Et_2O). Drying, evaporation and chromatography ($CHCl_3$ or $CHCl_3$/acetone, 10:1) or distillation gave the corresponding ethyl alkanoylacetate.

8.2.2 General method for the synthesis of ethyl α-alkylacetoacetates for the preparation of 3-alkyl-4-methylcoumarin sulphamates[242]

K_2CO_3 (2.4 eq.), water (50 ml), the alkyl bromide (1 eq.), ethyl acetoacetate (1 eq.), CH_2Cl_2 (50 ml/5g of alkyl bromide) and Bu_4NCl (1 or 2 eq.) were boiled under reflux for 3 d. After cooling, the separated organic layer was washed with 5M aq. HCl (30 ml). The mixture was extracted twice with Et_2O. The combined ethereal extracts were dried, filtered and concentrated *in vacuo*. Chromatography ($CHCl_3$ or $CHCl_3$/acetone 10:1) or distillation gave the corresponding ethyl α-alkylacetoacetate.

8.2.3 General method for the synthesis of ethyl 2-oxocycloalkylcarboxylates for the preparation of tricyclic coumarin sulphamates[258]

To a suspension of sodium hydride (60% dispersion in mineral oil) (NaH) (2 eq.) and diethyl carbonate (80 ml/5 g of cyclic ketone) under N_2 was added dropwise a solution of the corresponding cyclic ketone (1 eq.) in diethyl carbonate (20 ml) over a period of 0.5 h. When the evolution of H_2 had ceased (~15 h), the pale yellow solid residue was treated with H_2O and aq. HCl (20 ml). The mixture was extracted twice with Et_2O and H_2O. The combined ethereal extracts were dried and concentrated in *vacuo*. Chromatography ($CHCl_3$ or $CHCl_3$/acetone, 10:1) or distillation gave the corresponding ethyl 2-oxocycloalkyl carboxylates as oils.

8.2.4 General method for the synthesis of tricyclic, 3- or 4-alkyl or 3,4-dialkyl-7-hydroxycoumarins[219]

Resorcinol (1 eq.) was dissolved in the corresponding hot β-keto ester (1 eq.). The resulting syrup was cooled to 0°C and treated drop-wise with a mixture of CF_3COOH (2 eq.) and conc. H_2SO_4 (2 eq.) while keeping the temperature <10°C. After stirring for 3 h at R.T., the mixture was cautiously quenched with ice-water. The brightly coloured

gluey mass formed was stirred for further 1 h. The bright yellow/brown precipitate resulted was collected by suction filtration, washed exhaustively with water and re-dissolved in acetone. The yellow/brown solid obtained upon evaporation, was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and/or recrystallised from hot absolute ethanol, acetone/hexane (4:1) or THF/hexane (2:0.5) to give the corresponding coumarin as a crystalline solid.

8.2.5 General methods for the sulphamoylation reaction

Method A[196]

To a solution of the compound (1 eq.) in anhydrous DMF (5 ml) at 0°C under N_2 was added NaH (1 eq.). When the evolution of H_2 had ceased, previously prepared sulphamoyl chloride (~3-5 eq.) was introduced. After stirring at R.T. under N_2 overnight, the mixture was quenched with ice-water. The organic fractions were extracted into ethyl acetate (~150 ml) and washed with brine (4×100 ml). Drying, evaporation, chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and/or recrystallaisation with either ethyl acetate/hexane (5:2) or THF/hexane (2:1) gave the corresponding crystalline sulphamate.

Method B

To a solution of sulphamoyl chloride (~3-5 eq.) in toluene and *N,N*-dimethylacetamide (DMA) (1.5 eq.), the corresponding compound (1 eq.), was added at 0°C. After stirring at R.T. under N_2 overnight, the mixture was quenched with ice-water. The organic fractions were extracted into ethyl acetate (~150 ml) and washed with brine (4×100 ml). Drying, evaporation, chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and/or recrystallaisation with either ethyl acetate/hexane (5:2) or THF/hexane (2:1) gave the corresponding crystalline sulphamate.

8.3 Synthesis of compounds with varying lengths of alkyl chain at the C-4 position of the coumarin ring

8.3.1 Ethyl 3-oxoheptanoate (1)

Prepared by method A (8.2.1) using potassium ethylmalonate (12.6 g; 74.0 mmol), CH_3CN (110 ml), Et_3N (11.6 g; 115 mmol), $MgCl_2$ (8.39 g; 88.1 mmol) and pentanoyl chloride (4.34 g; 36.0 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **1** as a pale yellow oil (4.65 g; 78%). [398]Lit. bp_9 97-101°C); R_f: 0.92 ($CHCl_3$:acetone, 10:1); MS (FAB^+) *m/z*: 173.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 171.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 173.1089, $C_9H_{17}O_3$ requires 173.1099; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (t, 3H, 7-CH_3, J = 7.3 Hz), 1.28 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 1.29-1.37 (m, 2H, CH_2), 1.54-1.62 (m, 2H, CH_2), 2.55 (t, 2H, 4-CH_2, J = 7.3 Hz), 3.44 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.2 4-Butyl-7-hydroxycoumarin (2)

Prepared by method 8.2.4, using resorcinol (2.0 g; 18 mmol), **1** (3.13 g; 18.2 mmol) and a mixture of CF_3COOH (2.77 ml; 36.3 mmol) and conc. H_2SO_4 (1.83 ml; 36.3 mmol). The crude yellow/brown solid was recrystallised from acetone/hexane to give **2** as cream crystals (1.87 g; 47%). R_f: 0.63 ($CHCl_3$/acetone, 3:1); mp 135-138°C ([399]Lit. mp 139-140°C-ethanol); νmax (KBr) cm^{-1}: 3440 (OH), 1650 (C=O); MS (FAB^+) *m/z*: 437.2 [15, $(2M+H)^+$], 219.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 435.3 [20, $(2M-H)^-$], 217.2 [100, $(M-H^-)$; Acc. MS (FAB^+): 219.1034, $C_{13}H_{15}O_3$ requires 219.1021; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.92 (t, 3H, CH_3, J = 7.3 Hz), 1.34-1.43 (m, 2H, CH_2), 1.54-1.62 (m, 2H, CH_2), 2.73 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J= 2.4 Hz), 6.80 (dd, 1H, C_6-H, J = 8.6, 2.4 Hz), 7.6 (d, 1H, C_5-H, J = 8.5 Hz) and 10.53 (s, 1H, OH); Found C, 71.40; H, 6.49; $C_{13}H_{14}O_3$ requires C, 71.54; H, 6.47%.

8.3.3 4-Butylcoumarin-7-*O*-sulphamate (3)

Compound **2** (700 mg; 3.21 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was fractionated by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **3** as white crystals (98 mg; 10%). R_f: 0.36 ($CHCl_3$:ethyl acetate, 4:1); mp 147–150°C; νmax (KBr) cm^{-1}: 3400–3100 (N-H), 1750 (C=O), 1450-1300 (SO_2O), 1100-1150 (SO_2); MS (FAB^+) *m/z*: 595.2 [70. $(2M+H)^+$], 298.1 [100, $(M+H)^+$], 219.1 [10, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 593.2 [15, $(2M-H)^-$], 296.2 [100, $(M-H)^-$], 217.2 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 298.0742, $C_{13}H_{16}NO_5S$ requires 298.0749; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.93 (t, 3H, CH_3, J = 7.3 Hz), 1.36-1.45 (m, 2H, CH_2), 1.57-1.64 (m, 2H, CH_2), 2.82 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.38 (s, 1H, C_3-H), 7.29 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.33 (d, 1H, C_8-H, J = 2.4 Hz), 7.94 (d, 1H, C_5-H, J = 8.8 Hz) and 8.24 (s, 2H, NH_2); Found C, 52.00; H, 5.00; N, 4.61; $C_{13}H_{15}NO_5S$ requires C, 52.52; H, 5.09; N, 4.71%.

8.3.4 Ethyl 3-oxooctanoate (4)

Prepared by method A (8.2.1), using potassium ethylmalonate (13.0 g; 74.4 mmol), CH_3CN (120 ml), Et_3N (16.2 ml; 116 mmol), $MgCl_2$ (8.66 g; 90.1 mmol) and hexanoyl chloride (5.31 g; 38.2 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **4** as pale yellow oil (6.58 g; 93%). (^{400}Lit. bp_4 90-96°C); R_f: 0.88 ($CHCl_3$); MS (FAB^+) *m/z*: 187.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 185.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 187.1342, $C_{10}H_{19}O_3$ requires 187.1334; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.89 (t, 3H, CH_3, J = 7.1 Hz), 1.29 (t, 3H, $OCH_2C\underline{H}_3$, J = 7.3 Hz), 1.31-1.37 (m, 4H, CH_2CH_2), 1.56-1.63 (m, 2H, CH_2), 2.54 (t, 2H, 4-CH_2, J = 7.3 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $OC\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.5 7-Hydroxy-4-pentylcoumarin (5)

Prepared by method 8.2.4, using resorcinol (2.0 g; 18 mmol), **4** (3.4 g; 18 mmol) and a mixture of CF_3COOH (2.8 ml; 36 mmol) and conc. H_2SO_4 (1.8 ml; 36 mmol). The crude yellow/brown solid was recrystallised from acetone/hexane to give **5** as pale

yellow crystals (2.32 g; 56%). R_f: 0.86 ($CHCl_3$/acetone, 3:1); mp 148–150°C ([401]Lit. mp 145-146°C); MS (FAB^+) *m/z*: 465.3 [15, $(2M+H)^+$], 233.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 463.4 [10, $(2M-H)^-$], 231.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 233.1181, $C_{14}H_{17}O_3$ requires 233.1178; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.87 (t, 3H, 5'-CH_3, J = 7.1 Hz), 1.33-1.34 (m, 4H, CH_2CH_2), 1.58-1.61 (m, 2H, CH_2), 2.72 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.4 Hz), 6.80 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.64 (d, 1H, C_5-H, J = 8.8 Hz) and 10.53 (s, 1H, OH); Found C, 72.33; H, 6.96; $C_{14}H_{16}O_3$ requires C, 72.39; H, 6.94%.

8.3.6 4-Pentylcoumarin-7-*O*-sulphamate (6)

Compound **5** (700 mg; 3.01 mmol) was sulphamoylated by method A (8.2.5). The crude white sold (893 mg) was fractionated by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) to get a white solid, which was recrystallised from ethyl acetate/hexane to give **6** as white crystals (251 mg; 27%). R_f: 0.36 ($CHCl_3$/ethyl acetate, 4:1); mp 128–132°C; MS (FAB^+) *m/z*: 623.2 [70, $(2M+H)^+$], 312.1 [100, $(M+H)^+$], 233.1 [20, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 621.2 [20, $(2M-H)^-$], 310.2 [100, $(M-H)^-$], 231.2 [100, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 312.0908, $C_{14}H_{18}NO_5S$ requires 312.0906; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88 (t, 3H, 5'-CH_3, J = 7.1 Hz), 1.31-1.39 (m, 4H, CH_2CH_2), 1.59-1.64 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.37 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J = 1.5, 8.5 Hz), 7.33 (d, 1H, C_8-H, J = 1.5 Hz), 7.93 (d, 1H, C_5-H, J = 8.5 Hz) and 8.23 (s, 2H, NH_2); Found C, 54.70; H, 5.56; N, 4.50; $C_{14}H_{17}NO_5S$ requires C, 54.01; H, 5.50; N, 4.50%.

8.3.7 Ethyl 3-oxononanoate (7)

Prepared by method A (8.2.1), using ethyl potassium malonate (13 g; 74 mmol), CH_3CN (120 ml), Et_3N (16.2 g; 116 mmol), $MgCl_2$ (8.7 g; 91 mmol) and heptanoyl chloride (5.91 g; 36.2 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **7** as a pale yellow oil (4.51 g; 62%). ([402]Lit. bp_8 87°C); R_f: 0.64 ($CHCl_3$); MS (FAB^+) *m/z*: 201.2 [100, $(M+H)^+$]; Acc. MS (FAB^+):

201.1492, $C_{11}H_{21}O_3$ requires 201.1491; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 9-CH_3, J = 7.3 Hz), 1.26-1.32 (m, 9H, $CH_2C\underline{H}_3$ and 3×CH_2), 1.59 (m, 2H, 5-CH_2), 2.35 (t, 2H, 4-CH_2, J = 7.3 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.1 Hz).

8.3.8 4-Hexyl-7-hydroxycoumarin (8)

Prepared by method 8.2.4, using resorcinol (2.20 g; 19.9 mmol), **7** (4.0 g; 20 mmol) and a mixture of CF_3COOH (3.1 ml; 40 mmol) and conc. H_2SO_4 (2.04 ml; 39.9 mmol). The crude orange solid obtained was recrystallised from acetone/hexane to **8** as off-white crystals (2.95 g; 60%). R_f: 0.72 ($CHCl_3$/acetone, 3:1); mp 124–126°C; MS (FAB$^+$) *m/z*: 493.4 [10, $(2M+H)^+$], 247.2 [100, $(M+H)^+$]; MS (FAB$^-$) *m/z*: 491.3 [15, $(2M-H)^-$], 245.2 [100, $(M-H)^-$]; Acc. MS (FAB$^+$): 247.1334, $C_{15}H_{19}O_3$ requires 247.1334; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, 6'-CH_3, J = 7.1 Hz), 1.27-1.37 (m, 6H, 3×CH_2), 1.55-1.63 (m, 2H, CH_2), 2.72 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.4 Hz), 6.80 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.64 (d, 1H, C_5-H, J = 8.8 Hz) and 10.53 (s, 1H, OH); Found C, 73.30; H, 7.40; $C_{15}H_{18}O_3$ requires C, 73.15; H, 7.37%.

8.3.9 4-Hexylcoumarin-7-*O*-sulphamate (9)

Compound **8** (700 mg; 2.84 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **9** as fine white crystals (441 mg; 48%). R_f: 0.24 ($CHCl_3$/ethyl acetate, 4:1); mp 126–128°C; MS (FAB$^+$) *m/z*: 651.3 [10, $(2M+H)^+$], 326.2 [100, $(M+H)^+$], 247.2 [10, $(M+H-HNSO_2)^+$]; MS (FAB$^-$) *m/z*: 649.3 [15, $(2M-H)^-$], 324.2 [100, $(M-H)^-$], 245.2 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB$^+$): 326.1079, $C_{15}H_{20}NO_5S$ requires 326.1062; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 0.87 (t, 3H, 6'-CH_3, J = 6.7 Hz), 1.29-1.39 (m, 6H, 3×CH_2), 1.58-1.66 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.9 Hz), 6.37 (s, 1H, C_3-H), 7.29 (dd, 1H, C_6-H, J = 2.4, 8.6 Hz), 7.33 (d, 1H, C_8-H, J = 2.4 Hz), 7.93 (d, 1H, C_5-H,

J = 8.6 Hz) and 8.24 (s, 2H, NH_2); Found C, 55.20; H, 5.88; N, 4.25; $C_{15}H_{19}NO_5S$ requires C, 55.37; H, 5.89; N, 4.30%.

8.3.10 Ethyl 3-oxodecanoate (10)

Prepared by method A (8.2.1), using potassium ethylmalonate (10.5 g; 61.5 mmol), CH_3CN (120 ml), Et_3N (13.1 ml; 93.8 mmol), $MgCl_2$ (7.0 g; 73 mmol) and octanoyl chloride (5.0 ml g; 29 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **10** as a pale yellow oil (3.86 g; 61%). ([403]Lit. $bp_{0.3}$ 87-88°C); R_f: 0.76 ($CHCl_3$); MS (FAB^+) *m/z*: 215.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 213.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 215.1652, $C_{12}H_{23}O_3$ requires 215.1647); ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 10-CH_3, J = 7.3 Hz), 1.26-1.29 (m, 11H, $CH_2C\underline{H}_3$ and 4×CH_2), 1.57-1.61 (m, 2H, CH_2), 2.53 (t, 2H, 4-CH_2, J = 6.8 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.11 4-Heptyl-7-hydroxycoumarin (11)

Prepared according by method 8.2.4, using resorcinol (1.8 g; 16 mmol), **10** (3.5 g; 16 mmol) and a mixture of CF_3COOH (2.52 ml; 32.7 mmol) and conc. H_2SO_4 (1.67 ml; 32.7 mmol). The crude yellow solid was recrystallised from acetone/hexane to give **11** as yellow crystals (2.32 g; 55%). R_f: 0.78 ($CHCl_3$/acetone, 3:1); mp 106–107°C; MS (FAB^+) *m/z*: 261.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 519.3 [60, $(2M-H)^-$], 259.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 261.1501, $C_{16}H_{21}O_3$ requires 261.1491; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, 7'-CH_3, J = 7.1 Hz), 1.24-1.36 (m, 8H, 4×CH_2), 1.55-1.63 (m, 2H, CH_2), 2.72 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.4 Hz), 6.80 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.64 (d, 1H, C_5-H, J = 8.8 Hz) and 10.53 (s, 1H, OH); Found C, 73.60; H, 7.82; $C_{16}H_{20}O_3$ requires C, 73.82; H, 7.74%.

8.3.12 4-Heptylcoumarin-7-*O*-sulphamate (12)

Compound **11** (700 mg; 2.69 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) to get a white solid, which was recrystallised from ethyl acetate/hexane to give **12** as fine white crystals (503 mg; 55%). R_f: 0.42 ($CHCl_3$/ethyl acetate, 4:1); mp 137–139°C; MS (FAB^+) *m/z*: 679.3 [60, $(2M+H)^+$], 340.1 [100, $(M+H)^+$], 261.1 [10, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 677.1 [20, $(2M-H)^-$], 338.1 [100, $(M-H)^-$], 259.1 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 340.1215, $C_{16}H_{22}NO_5S$ requires 340.1218; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, 7'-CH_3, J = 7.1 Hz), 1.27-1.39 (m, 8H, 4×CH_2), 1.58-1.64 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.3 Hz), 6.37 (s, 1H, C_3-H), 7.27-7.33 (m, 2H, C_6-H and C_8-H), 7.39 (d, 1H, C_5-H, J = 8.8 Hz) and 8.22 (s, 2H, NH_2); Found C, 56.90; H, 6.31; N, 4.15; $C_{16}H_{21}NO_5S$ requires C, 56.62; H, 6.24; N, 4.13%.

8.3.13 Ethyl 3-oxoundecanoate (13)

Prepared by method A (8.2.1), using potassium ethylmalonate (13.0 g; 76.4 mmol), CH_3CN (120 ml), Et_3N (16.2 ml; 116 mmol), $MgCl_2$ (8.7 g; 91 mmol) and nonanoyl chloride (6.69 ml; 37.8 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **13** as pale yellow oil (6.73 g; 78%). ([404]Lit. $bp_{0.6}$ 116°C); R_f: 0.65 ($CHCl_3$); MS (FAB^+) *m/z*: 229.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 227.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 229.1794, $C_{13}H_{25}O_3$ requires 229.1725; ^1H NMR (400 MHz; $CDCl_3$); δ_H: 0.88 (t, 3H, 11-CH_3, J = 7.3 Hz); 1.26-1.61 (m, 15H, $CH_2C\underline{H}_3$ and 6×CH_2), 2.53 (t, 2H, 4-CH_2, J = 7.6 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.14 7-Hydroxy-4-octylcoumarin (14)

Prepared by method 8.2.4, using resorcinol (1.93 g; 17.5 mmol), **13** (4.0 g; 18 mmol) and a mixture of CF_3COOH (2.7 ml; 35 mmol) and conc. H_2SO_4 (1.8 ml; 35 mmol). The crude yellow solid was recrystallised from acetone/hexane to give **14** as yellow

crystals (2.31 g; 48%). R_f: 0.71 ($CHCl_3$/acetone, 3:1); mp 90–92°C; MS (FAB^+) *m/z*: 549.5 [80, $(2M+H)^+$], 275.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 547.4 [75, $(2M-H)^-$], 273.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 275.1647, $C_{17}H_{23}O_3$ requires 275.1647; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 8'-CH_3, J = 7.1 Hz), 1.25-1.36 (m, 10H, 5×CH_2), 1.55-1.62 (m, 2H, CH_2), 2.51 (t, 2H, 1'-H_2, J = 7.3 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J= 2.4 Hz), 6.80 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.64 (d, 1H, C_5-H, J = 8.8 Hz) and 10.53 (s, 1H, OH); Found C, 74.70; H, 8.18; $C_{17}H_{22}O_3$ requires C, 74.42; H, 8.08%.

8.3.15 4-Octylcoumarin-7-*O*-sulphamate (15)

Compound **14** (400 mg; 1.46 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) to get a white solid, which was recrystallised from ethyl acetate/hexane to give **15** as white fine crystals (279 mg; 53%). R_f: 0.37 ($CHCl_3$/ethyl acetate, 4:1); mp 124–125°C; MS (FAB^+) *m/z*: 707.0 [80, $(2M+H)^+$], 354.0 [100, $(M+H)^+$], 275.0 [20, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 705.2 [20, $(2M-H)^-$], 352.1 [100, $(M-H)^-$], 273.1 [70, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 354.1375, $C_{17}H_{24}NO_5S$ requires 354.1375; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 8'-CH_3, J = 6.7 Hz), 1.25-1.39 (m, 10H, 5×CH_2), 1.58-1.63 (m, 2H, CH_2), 2.81 (t, 2H, 1'-H_2, J = 7.3 Hz), 6.37 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J = 2.4, 8.5 Hz), 7.32 (d, 1H, C_8-H, J = 2.4 Hz), 7.93 (d, 1H, C_5-H, J = 8.5 Hz) and 8.24 (s, 2H, NH_2); Found C, 58.00; H, 6.50; N, 3.75; $C_{17}H_{23}NO_5S$ requires C, 57.77; H, 6.56; N, 3.96%.

8.3.16 Ethyl 3-oxododecanoate (16)

Prepared by method A (8.2.1), using potassium ethylmalonate (13.0 g; 76.4 mmol), CH_3CN (120 ml), Et_3N (16.2 ml; 116 mmol), $MgCl_2$ (8.7 g; 91 mmol) and decanoyl chloride (7.5 ml; 36 mol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **16** as a pale yellow oil (7.79 g; 89%). ([405]Lit. $bp_{0.15}$ 100-102°C); R_f: 0.75 ($CHCl_3$); MS (FAB^+) *m/z*: 243.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 241.1 [100, (M-

H)$^-$]; Acc. MS (FAB$^+$): 243.1959, $C_{14}H_{27}O_3$ requires 243.1960; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.82 (t, 3H, 12-CH_3, J = 5.5 Hz), 1.19-1.55 (m, 17H, $CH_2C\underline{H}_3$ and 7×CH_2), 2.48 (t, 2H, 4-CH_2, J = 7.3 Hz), 3.37 (s, 2H, 2-CH_2) and 4.13 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.17 7-Hydroxy-4-nonylcoumarin (17)

Prepared by method 8.2.4, using resorcinol (1.14 g; 10.3 mmol), **16** (2.5 g; 10 mmol) and a mixture of CF_3COOH (1.6 ml; 21 mmol) and conc. H_2SO_4 (1.05 ml; 20.6 mmol). The crude yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **17** as off-white fine crystals (532 mg; 18%). R_f: 0.71 ($CHCl_3$/acetone, 3:1); mp 91–93°C; MS (FAB$^+$) *m/z*: 577.2 [80, (2M+H)$^+$], 289.1 [100, (M+H)$^+$]; MS (FAB$^-$) *m/z*: 575.2 [20, (2M-H)$^-$], 287.1 [100, (M-H)$^-$]; Acc. MS (FAB$^+$): 289.1807, $C_{18}H_{25}O_3$ requires 289.1804; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 9'-CH_3, J = 7.1 Hz), 1.27-1.44 (m, 12H, 6×CH_2), 1.64-1.72 (m, 2H, CH_2), 2.73 (t, 2H, 1'-CH_2, J = 7.3 Hz), 6.14 (s, 1H, C_3-H), 6.88 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.08 (d, 1H, C_8-H, J = 2.4 Hz), 7.52 (d, 1H, C_5-H, J = 8.8 Hz) and 10.54 (s, 1H, OH); Found C, 75.10; H, 8.39; $C_{18}H_{24}O_3$ requires C, 74.97; H, 8.39%.

8.3.18 4-Nonylcoumarin-7-*O*-sulphamate (18)

Compound **17** (400 mg; 1.39 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **18** as white fine crystals (36 mg; 7%). R_f: 0.40 ($CHCl_3$/ethyl acetate, 4:1); mp 101–103°C; MS (FAB$^+$) *m/z*: 735.3 [90, (2M+H)$^+$], 368.1 [100, (M+H)$^+$]; MS (FAB$^-$) *m/z*: 733.1 [80, (2M-H)$^-$], 366.0 [100, (M-H)$^-$], 287.1 [90, (M-H_2NSO_2)$^-$]; Acc. MS (FAB$^+$): 368.1539, $C_{18}H_{26}NO_5S$ requires 368.1532; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 9'-CH_3, J = 7.1 Hz), 1.18-1.43 (m, 12H, 6×CH_2), 1.57-1.65 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.3 Hz), 6.37 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J = 2.4,

8.5 Hz), 7.33 (d, 1H, C_8-H, J = 2.4 Hz), 7.93 (d, 1H, C_5-H, J = 8.5 Hz) and 8.24 (s, 2H, NH_2); Found C, 59.05; H, 6.91; N, 3.74; $C_{18}H_{25}NO_5S$ requires C, 58.84; H, 6.86; N, 3.81%.

8.3.19 Ethyl 3-oxotridecanoate (19)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (3.52 g; 30.8 mmol), $SnCl_2$ (556 mg; 2.9 mmol) and undecanal (5.0 g; 29 mmol) in CH_2Cl_2. The crude oily residue was purified by fractional distillation under reduced pressure to give **19** as a pale yellow oil (4.34 g; 58%). R_f: 0.72 ($CHCl_3$); $bp_{0.15}$ 135–139°C. ([406]Lit. $bp_{0.15}$ 130-135°C); MS (FAB^+) *m/z*: 257.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 255.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 257.2129, $C_{15}H_{29}O_3$ requires 257.2117; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 13-CH_3, J = 6.9 Hz), 1.26-1.61 (m, 19H, $CH_2C\underline{H}_3$ and 8×CH_2), 2.53 (t, 2H, 4-CH_2, J = 7.2 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.2 Hz).

8.3.20 4-Decyl-7-hydroxycoumarin (20)

Prepared according to method 8.2.4, using resorcinol (1.07 g; 9.76 mmol), **19** (2.5 g; 9.8 mmol) and a mixture of CF_3COOH (1.5 ml; 20 mmol) and conc. H_2SO_4 (1.0 ml; 20 mmol). The crude yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallisation from acetone/hexane to give **20** as off-white fine crystals (1.66 g; 54%). R_f: 0.73 ($CHCl_3$/acetone, 3:1); mp 98–99°C; MS (FAB^+) *m/z*: 303.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 301.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 303.1973, $C_{19}H_{27}O_3$ requires 303.1960; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 10'-CH_3, J = 7.0 Hz), 1.27-1.42 (m, 14H, 7×CH_2), 1.64-1.72 (m, 2H, CH_2), 2.73 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.14 (s, 1H, C_3-H), 6.89 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.11 (d, 1H, C_8-H, J = 2.4 Hz), 7.52 (d, 1H, C_5-H, J = 8.8 Hz) and 8.19 (s, 1H, OH); Found C, 75.10; H, 8.72; $C_{19}H_{26}O_3$ requires C, 75.46; H, 8.67%.

8.3.21 4-Decylcoumarin-7-*O*-sulphamate (21)

Compound **20** (400 mg; 1.32 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was fractionated by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **21** as white fine needles (288 mg; 57%). R_f: 0.55 ($CHCl_3$/ethyl acetate, 4:1); mp 112–115°C; MS (FAB^+) *m/z*: 763.2 [65, $(2M+H)^+$], 382.0 [100, $(M+H)^+$], 303.1 [20, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 761.0 [80, $(2M-H)^-$], 380.0 [100, $(M-H)^-$], 301.1 [90, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 382.1696, $C_{19}H_{28}NO_5S$ requires 382.1688, ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 10'-CH_3, J = 7.1 Hz), 1.16-1.38 (m, 14H, 7×CH_2), 1.58-1.63 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.37 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J = 2.1, 8.8 Hz), 7.33 (d, 1H, C_8-H, J = 2.1 Hz), 7.93 (d, 1H, C_5-H, J = 8.8 Hz) and 8.24 (s, 2H, NH_2); Found C, 60.15; H, 7.12; N, 3.54; $C_{19}H_{27}NO_5S$ requires C, 59.82; H, 7.13; N, 3.67%.

8.3.22 Ethyl 3-oxotetradecanoate (22)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (3.25 g; 28.5 mmol), $SnCl_2$ (514 mg; 2.7 mmol) and dodecyl aldehyde (5.0 g; 27 mmol) in CH_2Cl_2 (20 ml). The crude oily residue was purified by fractional distillation under reduced pressure to give **22** as a colourless oil (5.3 g; 72%). R_f: 0.74 ($CHCl_3$); $bp_{0.15}$ 122–123°C. ([407]Lit. $bp_{0.1}$ 123-125°C); MS (FAB^+) *m/z*: 271.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 269.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 271.2285, $C_{16}H_{31}O_3$ requires 271.2273; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 14-CH_3, J = 7.0 Hz), 1.25-1.61 (m, 21H, $CH_2C\underline{H}_3$ and 9×CH_2), 2.53 (t, 2H, 4-CH_2, J = 7.3 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.23 7-Hydroxy-4-undecylcoumarin (23)

Prepared by method 8.2.4, using resorcinol (1.22 g; 11.1 mmol), **22** (3.0 g; 11 mmol) and a mixture of CF_3COOH (2.0 ml; 22 mmol) and conc. H_2SO_4 (2.0 ml; 22 mmol). The crude yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to

4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **23** as white crystals (1.92 g; 55%). R_f: 0.86 ($CHCl_3$/acetone, 3:1); mp 102-105°C; MS (FAB^+) *m/z*: 317.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 631.4 [10, $(2M-H)^-$], 315.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 317.2121, $C_{20}H_{29}O_3$ requires 317.2117; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 11'-CH_3, J = 6.2 Hz), 1.23-1.36 (m, 16H, 8×CH_2), 1.54-1.60 (m, 2H, CH_2), 2.72 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.07 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.1 Hz), 6.79 (dd, 1H, C_6-H, J = 2.1, 8.5 Hz), 7.63 (d, 1H, C_5-H, J = 8.5 Hz) and 10.52 (s, 1H, OH); Found C, 75.50; H, 8.97; $C_{20}H_{28}O_3$ requires C, 75.91; H, 8.92%.

8.3.24 4-Undecylcoumarin-7-*O*-sulphamate (24)

Compound **23** (400 mg; 1.27 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was fractionated by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **24** as white fine fluffy crystals (88 mg; 18%). R_f: 0.54 ($CHCl_3$/ethyl acetate, 4:1); mp 114–116°C; MS (FAB^+) *m/z*: 396.1 [100, $(M+H)^+$], 317.2 [20, $(M+H-HNSO_2)^-$]; MS (FAB^-) *m/z*: 394.3 [100, $(M-H)^-$], 315.3 70, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 396.1843, $C_{20}H_{30}NO_5S$ requires 396.1845; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 11'-CH_3, J = 7.0 Hz), 1.24-1.38 (m, 16H, 8×CH_2), 1.58-1.65 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.4 Hz), 6.37 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J_6 = 2.3, 8.9 Hz), 7.33 (d, 1H, C_8-H, J = 2.3 Hz), 7.93 (d, 1H, C_5-H, J = 8.6 Hz) and 8.24 (s, 2H, NH_2); Found C, 61.00; H, 7.44; N, 3.52; $C_{20}H_{29}NO_5S$ requires C, 60.74; H, 7.39; N, 3.54%.

8.3.25 Ethyl 3-oxopentadecanoate (25)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (3.02 g; 26.5 mmol), $SnCl_2$ (478 mg; 2.5 mmol) and tridecanal (5.0 g; 25 mmol) in CH_2Cl_2 (20 ml). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **25** as a colourless oil, which solidified to a white soft solid on standing (6.98 g; 97%). R_f: 0.65

($CHCl_3$); mp 28°C ([406]Lit. mp <20°C); MS (FAB^+) *m/z*: 285.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 283.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 285.2426, $C_{17}H_{33}O_3$ requires 285.2429; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 15-CH_3, J = 6.4 Hz), 1.25-1.64 (m, 23H, $CH_2C\underline{H}_3$ and 10×CH_2), 2.53 (t, 2H, 4-CH_2, J = 7.3 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.26 4-Dodecyl-7-hydroxycoumarin (26)

Prepared by method 8.2.4, using resorcinol (1.16 g; 10.5 mmol), **25** (3.0 g; 11 mmol) and a mixture of CF_3COOH (2.0 ml; 21 mmol) and conc. H_2SO_4 (1.5 ml; 21 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the yellow solid isolated was recrystallised from acetone/hexane to give **26** as fine yellow crystals (747 mg; 22%). R_f: 0.77 ($CHCl_3$/acetone, 3:1); mp 101-103°C; MS (FAB^+) *m/z*: 331.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 329.3 [100, $(M-H)^+$]; Acc. MS (FAB^+): 331.2279, $C_{21}H_{31}O_3$ requires 330.2273; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 12'-CH_3, J = 7.0 Hz), 1.17-1.36 (m, 18H, 9×CH_2), 1.55-1.60 (m, 2H, CH_2), 2.72 (t, 2H, 1'-CH_2, J = 7.4 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.3 Hz), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.64 (d, 1H, C_5-H, J = 8.9 Hz) and 10.54 (s, 1H, OH); Found C, 76.80; H, 8.80; $C_{21}H_{30}O_3$ requires C, 76.33; H, 9.15%.

8.3.27 4-Dodecylcoumarin-7-*O*-sulphamate (27)

Compound **26** (400 mg; 1.21 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **27** as white fine crystals (91 mg; 18%). R_f: 0.55 ($CHCl_3$/ethyl acetate, 4:1); mp 104-106°C; MS (FAB^+) *m/z*: 410.2 [100, $(M+H)^+$], 331.2 [20, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 817.3 [20, $(2M-H)^-$], 408.3 [100, $(M-H)^-$], 329.3 [70, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 410.2006, $C_{21}H_{32}NO_5S$ requires 410.2001; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 12'-CH_3, J = 7.0 Hz), 1.24-1.34 (m, 18H, 9×CH_2), 1.58-1.63 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.37 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J =

2.3, 8.9 Hz), 7.33 (d, 1H, C_8-H, J = 2.3 Hz), 7.93 (d, 1H, C_5-H, J = 8.6 Hz) and 8.23 (s, 2H, NH_2); Found C, 61.90; H, 7.65; N, 3.34; $C_{21}H_{31}NO_5S$ requires C, 61.59; H, 7.63; N, 3.42%.

8.3.28 Ethyl 3-oxohexadecanoate (28)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (2.82 g; 24.7 mmol), $SnCl_2$ (446 mg; 2.35 mmol) and tetradecanal (5.0 g; 24 mmol) in CH_2Cl_2 (20 ml). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **28** as a colourless oil, which solidified to an off-white soft solid on standing (5.45 g; 78%). R_f: 0.71 ($CHCl_3$); mp 45-47°C ([408]Lit. mp 41-42°C); MS (FAB^+) *m/z*: 299.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 297.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 299.2599, $C_{18}H_{35}O_3$ requires 299.2586; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, 16-CH_3, J = 6.67 Hz), 1.20-1.69 (m, 25H, $CH_2C\underline{H}_3$ and 11×CH_2), 2.53 (t, 2H, 4-cH_2, J = 7.3 Hz), 3.43 (s, 2H, 2-CH_2) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.3.29 7-Hydroxy-4-tridecylcoumarin (29)

Prepared by method 8.2.4, using resorcinol (1.11 g; 10.1 mmol), **28** (3.0 g; 10 mmol) and a mixture of CF_3COOH (2.0 ml; 20 mmol) and conc. H_2SO_4 (1.5 ml; 20 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the beige solid isolated was recrystallised from acetone/hexane to give **29** as fine cream-coloured crystals (757 mg; 22%). R_f: 0.72 ($CHCl_3$/acetone, 3:1); mp 95-97°C; MS (FAB^+) *m/z*: 345.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 343.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 345.2438, $C_{22}H_{33}O_3$ requires 345.2429; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, 13'-CH_3, J = 7.0 Hz), 1.19-1.36 (m, 20H, 10×CH_2), 1.54-1.62 (m, 2H, CH_2), 2.72 (t, 2H, 1'-CH_2, J = 7.4 Hz), 6.08 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.3 Hz), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.9 Hz), 7.64 (d, 1H, C_5-H, J = 8.6 Hz) and 10.54 (s, 1H, OH); Found C, 76.85; H, 9.31; $C_{22}H_{32}O_3$ requires C, 76.70; H, 9.36%.

8.3.30 4-Tridecylcoumarin-7-*O*-sulphamate (30)

Compound **29** (350 mg; 1.01 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **30** as white crystals (101 mg; 23%). R_f: 0.53 ($CHCl_3$/ethyl acetate, 4:1); mp 120-121°C; MS (FAB^+) *m/z*: 424.3 [100, $(M+H)^+$], 345.3 [25, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 422.3 [60, $(M-H)^-$], 343.3 [100, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 424.2168, $C_{22}H_{34}NO_5S$ requires 424.2158; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, 13'-CH_3, J = 7.0 Hz), 1.20-1.39 (m, 20H, 10×CH_2), 1.58-1.66 (m, 2H, CH_2), 2.81 (t, 2H, 1'-CH_2, J = 7.8 Hz), 6.38 (s, 1H, C_3-H), 7.29 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.33 (d, 1H, C_8-H, J = 2.3 Hz), 7.93 (d, 1H, C_5-H, J = 8.9 Hz) and 8.24 (s, 2H, NH_2); Found C, 62.60; H, 7.90; N, 3.46; $C_{22}H_{33}NO_5S$ requires C, 62.38; H, 7.85; N, 3.31%.

8.4 Synthesis of compounds with other functionalities at the C-4 position of the coumarin ring

8.4.1 7-Hydroxy-4-(prop-2-yl)coumarin (31)

Prepared by method 8.2.4, using resorcinol (1.21 g; 11.1 mmol), ethyl 4-methyl-3-oxopentanoate (1.6 g; 10 mmol) and a mixture of CF_3COOH (1.7 ml; 22 mmol) and conc. H_2SO_4 (2.2 ml; 22 mmol). The crude yellow solid was purified by recrystallisation from acetone/hexane to give **31** as pale white fine crystals (420 mg; 21%). R_f: 0.42 ($CHCl_3$/acetone 3:1); mp 120–122°C ([409]Lit. mp 62-64°C); MS (FAB^+) *m/z*: 409.2 [20, $(2M+H)^+$], 205.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 407.2 [20, $(2M-H)^-$], 203.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 205.0874, $C_{12}H_{13}O_3$ requires 205.0865; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.24 (d, 6H, $CH(C\underline{H}_3)_2$, J = 6.7 Hz), 3.28-3.35 (m, 1H, $C\underline{H}(CH_3)_2$), 6.08 (s, 1H, C_3-H), 6.72 (d, 1H, C_8-H, J = 2.1 Hz), 6.81 (dd, 1H, C_6-H, J = 2.1, 8.8 Hz), 7.71 (d, 1H, C_5-H, J = 8.8 Hz) and 10.54 (s, 1H, OH); Found C, 70.60; H, 6.00; $C_{12}H_{12}O_3$ requires C, 70.57; H, 5.92%.

8.4.2 4-(Prop-2-yl)coumarin-7-*O*-sulphamate (32)

Compound **31** (400 mg; 1.96 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **32** as white fine crystals (150 mg; 30%). R_f: 0.22 ($CHCl_3$/ethyl acetate, 4:1); mp 164–167°C; MS (FAB^+) *m/z*: 567.1 [70, $(2M+H)^+$], 284.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 565.2 [30, $(2M-H)^-$], 282.1 [100, $(M-H)^-$], 203.1 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 284.0599, $C_{12}H_{14}NO_5S$ requires 284.0593; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.26 (d, 6H, $CH(C\underline{H}_3)_2$, J = 6.7 Hz), 3.31-3.34 (m, 1H, $C\underline{H}(CH_3)_2$), 6.35 (s, 1H, C_3-H), 7.29 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.33 (d, 1H, C_8-H, J = 2.4 Hz), 7.99 (d, 1H, C_5-H, J = 8.8 Hz) and 8.23 (s, 2H, NH_2); Found C, 50.80; H, 4.62; N, 4.97 $C_{12}H_{13}NO_5S$ requires C, 50.88; H, 4.63; N, 4.94%.

8.4.3 7-Hydroxy-4-^{t}butylcoumarin (33)

Prepared by method 8.2.4, using resorcinol (7.0 g; 63 mmol), methyl 4,4-dimethyl-3-oxopentanoate (10.0 g; 63.2 mmol) and a mixture of CF_3COOH (10 ml; 0.2 mol) and conc. H_2SO_4 (6.5 ml; 0.2 mol). The crude brown residue was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from THF/hexane to give **33** as pale yellow crystals (380 mg; 0.03%). R_f: 0.61 ($CHCl_3$/acetone, 3:1); mp 159-161°C; MS (FAB^+) *m/z*: 219.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 217.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 219.1029, $C_{13}H_{15}O_3$ requires 219.1021; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.49 (s, 9H, $C(CH_3)_3$), 6.28 (s, 1H, C_3-H), 6.85 (dd, 1H, C_6-H, J = 2.7, 8.9 Hz), 7.11 (d, 1H, C_8-H, J = 2.7 Hz), 7.13 (s, 1H, OH) and 7.91 (d, 1H, C_5-H, $J_{6,5}$ = 8.9 Hz); Found C, 71.90; H, 6.49; $C_{13}H_{14}O_3$ requires C, 71.54; H, 6.47%; HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 321 nm; t_R = 1.91 min.

8.4.4 4-^{t}Butylcoumarin-7-*O*-sulphamate (34)

Compound **33** (310 mg; 1.43 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl THF/hexane to give **34** as white fine crystals (43 mg; 10%). R_f: 0.48 ($CHCl_3$/ethyl acetate, 4:1); mp 187-189°C; MS (FAB^+) *m/z*: 298.0 [100, $(M+H)^+$], 219.1 [15, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 296.0 [100, $(M-H)^-$], 217.0 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 298.0746, $C_{13}H_{16}NO_5S$ requires 298.0749; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.45 (s, 9H, $C(CH_3)_3$), 6.33 (s, 1H, C_3-H), 7.27 (dd, 1H, C_6-H, J = 2.3, 8.9 Hz), 7.34 (d, 1H, C_8-H, J = 2.3 Hz), 8.26 (s, 2H, NH_2) and 8.28 (d, 1H, C_5-H, J = 8.9 Hz); Found C, 52.40; H, 4.91; N, 4.76; $C_{13}H_{15}O_5NS$ requires C, 52.51; H, 5.08; N, 4.71%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 273.4 and 310.1 nm; t_R = 1.6 min.

8.4.5 7-Hydroxy-4-(methoxymethyl)coumarin (35)

Prepared by method 8.2.4, using resorcinol (3.1 g; 28 mmol), methyl 4-methoxy-3-oxobutanoate (4.0 g; 28 mmol) and a mixture of CF_3COOH (4.34 ml; 56.3 mmol) and conc. H_2SO_4 (2.85 ml; 56.3 mmol). The crude brown solid was purified by recrystallisation from acetone/hexane to give **35** as pale pink fine crystals (1.52 g; 27%). R_f: 0.54 ($CHCl_3$/acetone, 3:1); mp 210–212°C; MS (FAB^+) *m/z*: 413.2 [10, $(2M+H)^+$], 207.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 512.2 [10, $(2M-H)^-$], 205.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 207.0641, $C_{11}H_{11}O_4$ requires 207.0657; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 3.41 (s, 3H, CH_3), 4.63 (s, 2H, CH_2O), 6.17 (s, 1H, C_3-H), 6.73 (d, 1H, C_8-H, J = 2.4 Hz), 6.79 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.53 (d, 1H, C_5-H, J = 8.8 Hz) and 10.57 (s, 1H, OH); Found C, 64.30; H, 4.90; $C_{11}H_{10}O_4$ requires C, 64.07; H, 4.89%.

8.4.6 4-(Methoxymethyl)coumarin-7-*O*-sulphamate (36)

Compound **35** (was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to

give **36** as fine fluffy white crystals (301 mg; 31%). R_f: 0.14 ($CHCl_3$/ethyl acetate, 4:1); mp 151–153°C; MS (FAB^+) *m/z*: 571.2 [10, $(2M+H)^+$], 286.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 284.1 [100, $(M-H)^-$], 205.1 [50, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 286.0384, $C_{11}H_{12}NO_6S$ requires 286.0385; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 3.43 (s, 3H, CH_3), 4.71 (s, 2H, CH_2O), 6.45 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J = 2.1, 8.7 Hz), 7.35 (d, 1H, C_8-H, J = 2.1 Hz), 7.81 (d, 1H, C_5-H, J = 8.5 Hz) and 8.25 (s, 2H, NH_2); Found C, 46.40; H, 3.84; N, 4.88; $C_{11}H_{11}NO_6S$ requires C, 46.31; H, 3.89; N, 4.91%.

8.4.7 4-Chloromethyl-7-hydroxycoumarin (37)

Prepared by method 8.2.4, using resorcinol (2.93 g; 29.6 mmol), methyl 4-chloro-3-oxobutanoate (4.0 g; 29 mmol) and a mixture of CF_3COOH (4.1 ml; 53 mmol) and conc. H_2SO_4 (2.7 ml; 53 mmol). The crude orange solid was purified by recrystallisation from acetone/hexane to give **37** as off-white fine crystals (1.31 g; 23%). R_f: 0.73 ($CHCl_3$/acetone, 3:1) mp 183–185°C ([410]Lit. mp 181°C); MS (FAB^+) *m/z*: 421.2 [15, $(2M+H)^+$], 211.1 [100, $(M(Cl^{35})+H)^+$]; MS (FAB^-) *m/z*: 419.1 [15, $(2M-H)^-$], 209.1 [100, $(M(Cl^{35})-H)^-$]; Acc. MS *m/z* (FAB^+): 213.0127, $C_{10}H_8{}^{37}ClO_3$ requires 213.0132 and 211.0151, $C_{10}H_8{}^{35}ClO_3$ requires 211.0162; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 4.96 (s, 2H, CH_2), 6.42 (s, 1H, C_3-H), 6.76 (d, 1H, C_8-H, J = 2.4 Hz), 6.85 (dd, 1H, C_6-H, J = 2.4, 8.7 Hz), 7.69 (d, 1H, C_5-H, J = 8.8 Hz) and 10.69 (s, 1H, OH); Found C, 57.00; H, 3.20; $C_{10}H_7ClO_3$ requires C, 57.03; H, 3.35%.

8.4.8 4-Chloromethylcoumarin-7-*O*-sulphamate (38)

Compound **37** (400 mg; 1.9 mmol) was sulphamoylated by method A (8.2.5). The crude yellow solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **38** as pale green fine crystals (59 mg; 11%). R_f: 0.44 ($CHCl_3$/ethyl acetate, 4:1); mp 172–175°C; MS (FAB^+) *m/z*: 289.9 [95, $(M(Cl^{35})+H)^+$], 210.9 [100, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 287.9 [100, $(M(Cl^{35})-H)]^-$, 208.9 [90, $(M-H_2NSO_2)^-$]; Acc. MS *m/z* (FAB^+): 289.9893, $C_{10}H_9{}^{35}ClNO_5S$ requires 289.9889 and 291.9862, $C_{10}H_9{}^{37}ClNO_5S$ requires 291.9860; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 5.05 (s, 2H,

CH_2), 6.72 (s, 1H, C_3-H), 7.34 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.38 (d, 1H, C_8-H, J = 2.4 Hz), 7.96 (d, 1H, C_5-H, J = 8.8 Hz) and 8.28 (s, 2H, NH_2); Found C, 41.50; H, 2.79; N, 3.48; $C_{10}H_8ClNO_5S$ requires C, 41.46; H, 2.78; N, 4.84%.

8.4.9 7-Hydroxy-4-phenylcoumarin (39)

Prepared by method 8.2.4, using resorcinol (2.0 g; 18 mmol), ethyl 3-oxo-3-phenylpropanoate (2.0 g; 18 mmol) and a mixture of CF_3COOH (2.8 ml; 36 mmol) and conc. H_2SO_4 (1.85 ml; 36.3 mmol). The crude orange solid was purified by recrystallisation from hot absolute ethanol to give **39** as yellow crystals (1.85 g; 43%). R_f: 0.71 ($CHCl_3$/acetone, 3:1); mp 248–252°C ([411]Lit. mp 247-248°C); MS (FAB^+) *m/z*: 239.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 237.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 239.0634 (M+H) ($C_{15}H_{11}O_3$ requires 239.0629); ^1H NMR (400 MHz; DMSO-d_6) δ_H: 6.15 (s, 1H, C_3-H), 6.79 (dd, 1H, C_6-H, J = 1.2, 8.8 Hz), 6.81 (d, 1H, C_8-H, J = 1.2 Hz), 7.27 (d, 1H, C_5-H, J = 8.8 Hz), 7.51-7.57 (m, 5H, Ph-H) and 10.67 (s, 1H, OH); Found C, 75.40; H, 4.13; $C_{15}H_{10}O_3$ requires C, 75.62; H, 4.23%.

8.4.10 4-Phenylcoumarin-7-*O*-sulphamate (40)

Compound **39** (700 mg; 2.94 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **40** as white fine fluffy crystals (304 mg; 33%). R_f: 0.60 ($CHCl_3$/ethyl acetate, 4:1); mp 185–190°C; MS (FAB^+) *m/z*: 318.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 316.2 [100, $(M-H)^-$], 237.2 [65, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 318.0379 (M+H) ($C_{15}H_{12}NO_5S$ requires 318.0357); ^1H NMR (400 MHz; DMSO-d_6) δ_H: 6.36 (s, 1H, C_3-H), 7.28 (dd, 1H, C_6-H, J = 2.1, 8.7 Hz), 7.39 (d, 1H, C_8-H, J = 2.1 Hz), 7.58 (d, 1H, C_5-H, J = 8.7 Hz) and 7.59-7.62 (m, 7H, Ph-H and NH_2 – reduced to 5H when exchanged with D_2O); Found C, 56.70; H, 3.53; N, 4.48; $C_{15}H_{11}NO_5S$ requires C, 56.78; H, 3.49; N, 4.41%.

8.4.11 Ethyl 3-oxo-4-phenylbutanoate (41)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (4.99 g; 43.7 mmol), $SnCl_2$ (790 mg; 3.73 mmol) and phenylacetaldehyde (5.0 g; 42 mmol) in CH_2Cl_2. The crude oily residue was purified by distillation under reduced pressure to give **41** as a pale yellow oil (5.27 g; 61%). $bp_{0.3}$ 185-189°C. ([412]Lit. bp_9 154–156°C); R_f: 0.62 ($CHCl_3$); MS (FAB^+) *m/z*: 207.1 [100, $(M+H)^+$], 91.1 [40, $(PhCH_2)^+$]; MS (FAB^-) *m/z*: 205.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 207.1014, $C_{12}H_{15}O_3$ requires 207.1021; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.16 (t, 3H, $CH_2C\underline{H}_3$, J = 7.3 Hz), 3.45 (s, 2H, Ph-$C\underline{H}_2$), 3.83 (s, 2H, 2-CH_2), 4.17 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz) and 7.20-7.36 (m, 5H, Ph-H).

8.4.12 4-Benzyl-7-hydroxycoumarin (42)

Prepared by method 8.2.4, using resorcinol (1.6 g; 15 mmol), **41** (3.0 g; 15 mmol) and a mixture of CF_3COOH (2.5 ml; 29 mmol) and conc. H_2SO_4 (1.5 ml; 29 mmol). The crude yellow solid was purified by recrystallisation from hot absolute ethanol to give **42** as pale yellow crystals (2.19 g; 60%). R_f: 0.80 ($CHCl_3$/acetone, 3:1); mp 209-212°C ([413]Lit. mp 214-215°C); MS (FAB^+) *m/z*: 505.1 [10, $(2M+H)^+$], 253.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 503.2 [15, $(2M-H)^-$], 251.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 253.0863, $C_{16}H_{13}O_3$ requires 253.0865; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 4.38 (s, 2H, $C\underline{H}_2$-Ph), 5.98 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.3 Hz), 6.76 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.23-7.36 (m, 5H, Ph-H), 7.67 (d, 1H, C_5-H, J = 8.6 Hz) and 10.57 (s, 1H, OH); Found C, 75.60; H, 4.88; $C_{16}H_{12}O_3$ requires C, 76.18; H, 4.79%.

8.4.13 4-Benzylcoumarin-7-*O*-sulphamate (43)

Compound **42** (400 mg; 1.6 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **43** as white fine crystals (135 mg; 26%). R_f: 0.39 ($CHCl_3$/ethyl acetate, 4:1); mp 180-182°C; MS (FAB^+) *m/z*: 663.4 [30, $(2M+H)^+$], 332.1 [100, $(M+H)^+$]; MS (FAB^-)

m/z: 330.2 [100, $(M-H)^-$], 251.2 [50, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 332.0590 (M+H) ($C_{16}H_{14}NO_5S$ requires 332.0593); ^1H NMR (400 MHz; DMSO-d_6) δ_H: 4.23 (s, 2H, C<u>H</u>$_2$-Ph), 6.32 (s, 1H, C_3-H), 7.23-7.26 (m, 2H, C_8-H and C_6-H), 7.26-7.37 (m, 5H, Ph-H), 7.92 (d, 1H, C_5-H, J = 8.9 Hz) and 8.22 (s, 2H, NH_2); Found C, 57.70; H, 3.97; N, 4.21; $C_{16}H_{13}NO_5S$ requires C, 58.00; H, 3.95; N, 4.23%.

8.4.14 Ethyl 3-oxo-5-phenylpentanoate (44)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (4.5 g; 39 mmol), $SnCl_2$ (700 mg; 3.73 mmol) and hydrocinnamaldehyde (5.0 g; 37 mmol) in CH_2Cl_2. The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **44** as a pale yellow oil (5.38 g; 66%). ([414]Lit. $bp_{0.1}$ 114-122°C); R_f: 0.63 ($CHCl_3$); MS (FAB^+) *m/z*: 221.1 [100, $(M+H)^+$], 91.0 [55, $(PhCH_2)^+$]; MS (FAB^-) *m/z*: 219.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 221.1181 (M+H) ($C_{13}H_{17}O_3$ requires 221.1178); ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.17 (t, 3H, $CH_2C\underline{H}_3$, J = 7.3 Hz), 2.79 (t, 2H, 4-CH_2, J = 7.5 Hz), 2.87 (t, 2H, 5-CH_2, J = 7.5 Hz), 3.60 (s, 2H, 2-H_2), 4.08 (q, 2H, C<u>H</u>$_2$CH$_3$, J = 7.3 Hz) and 7.13-7.29 (m, 5H, Ph-H).

8.4.15 7-Hydroxy-4-(2-phenylethyl)coumarin (45)

Prepared by method 8.2.4, using resorcinol (1.25 g; 11.4 mmol), **44** (2.5 g; 11 mmol) and a mixture of CF_3COOH (1.75 ml; 22.7 mmol) and conc. H_2SO_4 (1.16 ml; 22.7 mmol). The crude yellow solid was purified by recrystallisation from hot absolute ethanol to give **45** as pale white crystals (1.06 g; 35%). R_f: 0.65 ($CHCl_3$/acetone, 3:1); mp 175–177°C ([415]Lit. mp 175-176°C); MS (FAB^+) *m/z*: 533.2 [40, $(2M+H)^+$], 267.1 [100, $(M+H)^+$], 91.1 [20, $(CH_2Ph)^+$]; MS (FAB^-) *m/z*: 531.2 [30, $(2M-H)^-$], 265.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 267.1018, $C_{17}H_{15}O_3$ requires 267.1021; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.98-3.06 (m, 4H, CH_2CH_2), 6.12 (s, 1H, C_3-H), 6.59 (s, 1H, OH), 6.84 (dd, 1H, C_6-H, J= 2.4, 8.8 Hz), 7.11 (d, 1H, C_8-H, J = 2.4 Hz), 7.20-7.34 (m, 5H, Ph-H) and 7.53 (d, 1H, C_5-H, J = 8.8 Hz); Found C, 76.70; H, 5.20; $C_{17}H_{14}O_3$ requires C, 76.68; H, 5.30%.

8.4.16 4-(2-Phenylethyl)coumarin-7-*O*-sulphamate (46)

Compound **45** (400 mg; 1.5 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **46** as white fine crystals (395 mg; 76%). R_f: 0.27 ($CHCl_3$/ethyl acetate, 4:1); mp 89–93°C; MS (FAB^+) *m/z*: 346.0 [100, $(M+H)^+$], 91.0 [50, $(CH_2Ph)^+$]; MS (FAB^-) *m/z*: 344.0 [100, $(M-H)^-$], 265.0 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 346.0754, $C_{17}H_{16}NO_5S$ requires 346.0749; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.96 (t, 2H, $C\underline{H}_2CH_2Ph$, J = 8.8 Hz), 3.14 (t, 2H, CH_2Ph, J = 8.5 Hz), 6.36 (s, 1H, C_3-H), 7.22-7.34 (m, 7H, Ph-H, C_8-H and C_6-H), 8.03 (d, 1H, C_5-H, J = 7.9 Hz) and 8.25 (s, 2H, NH_2); Found C, 59.30; H, 4.89; N, 3.86; $C_{17}H_{15}NO_5S$ requires C, 59.12; H, 4.38; N, 4.06%.

8.4.17 Ethyl 3-(4-ethylphenyl)-3-oxopropanoate (47)

Prepared by method A (8.2.1), using ethyl potassium malonate (6.07 g; 35.7 mmol), CH_3CN (120 ml), Et_3N (7.58 ml; 54.4 mmol), $MgCl_2$ (4.05 g; 42.5 mmol) and 4-ethylbenzoyl chloride (2.5 ml; 17 mmol). The crude oily residue was purified by distillation under reduced pressure to give **47** as colourless oil (3.74 g; 78%). R_f: 0.64 (CH_2Cl_2); $bp_{0.23}$ 131-135°C. ([416]Lit. $bp_{0.004}$ 105-112°C); MS (FAB^+) *m/z*: 221.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 219.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 221.1177, $C_{13}H_{17}O_3$ requires 221.1178; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.22-1.33 (m, 6H, $C\underline{H}_3CH_2Ph$ and $CH_2C\underline{H}_3$), 2.70 (q, 2H, CH_2Ph, J = 7.8 Hz), 3.97 (s, 2H, 2-H_2), 4.21 (q, 2H, $C\underline{H}_2CH_3$, J = 6.8 Hz), 7.29 (d, 2H, Ar-H_2, J = 8.3 Hz) and 7.87 (d, 2H, Ar-H_2, J = 8.3 Hz).

8.4.18 4-(4-Ethylphenyl)-7-hydroxycoumarin (48)

Prepared by method 8.2.4, using resorcinol (1.5 g; 14 mmol), **47** (3.0 g; 14 mmol) and a mixture of CF_3COOH (2.5 ml; 27 mmol) and conc. H_2SO_4 (1.5 ml; 27 mmol). The crude orange solid was purified by recrystallisation from hot ethanol to give **48** as white needles (1.66 g; 46%). R_f: 0.72 ($CHCl_3$/acetone, 4:1); mp 176-180°C; MS (FAB^+) *m/z*: 533.1 [10, $(2M+H)^+$], 267.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 531.1 [20,

(2M-H)$^-$], 265.1 [100, (M-H)$^-$]; Acc. MS (FAB$^+$): 267.1018, $C_{17}H_{15}O_3$ requires 267.1021; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 1.23 (t, 3H, C<u>H</u>$_3$CH$_2$, J= 7.6 Hz), 2.69 (q, 2H, CH$_3$C<u>H</u>$_2$, J= 7.6 Hz), 6.13 (s, 1H, C$_3$-H), 6.77 (m, 2H, C$_6$-H and C$_8$-H), 7.31 (d, 1H, C$_5$-H, J = 8.5 Hz), 7.38-7.44 (m, 4H, Ph-H) and 10.64 (s, 1H, OH); Found C, 76.30; H, 5.30; $C_{17}H_{14}O_3$ requires C, 76.68; H, 5.30%.

8.4.19 4-(4-Ethylphenyl)coumarin-7-*O*-sulphamate (49)

Compound **48** (400 mg; 1.5 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **49** as white fine crystals (114 mg; 22%). R_f: 0.35 ($CHCl_3$/ethyl acetate, 4:1); mp 170–173°C; MS (FAB$^+$) *m/z*: 691.0 [30, (2M+H)$^+$], 346.1 [100, (M+H)$^+$]; MS (FAB$^-$) *m/z*: 689.3 [10, (2M-H)$^-$], 344.2 [100, (M-H)$^-$], 265.2 [60, (M-H_2NSO_2)$^-$]; Acc. MS (FAB$^+$): 346.0749, $C_{17}H_{16}NO_5S$ requires 345.0749; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 1.25 (t, 3H, C<u>H</u>$_3$CH$_2$, J= 7.4 Hz), 2.71 (q, 2H, CH$_3$C<u>H</u>$_2$, J= 7.4 Hz), 6.44 (s, 1H, C$_3$-H), 7.26 (dd, 1H, C$_6$-H, J= 2.3, 8.9 Hz), 7.42-7.49 (m, 5H, Ph-H and C$_8$-H), 7.57 (d, 1H, C$_5$-H, J = 8.6 Hz) and 8.28 (s, 2H, NH$_2$); Found C, 59.00; H, 4.36; N, 4.03; $C_{17}H_{15}NO_5S$ requires C, 59.12; H, 4.38; N, 4.06%.

8.4.20 Ethyl 3-cyclohexyl-3-oxopropanoate (50)

Prepared by method B (8.2.1), using CH_2Cl_2 (80 ml), ethyl diazoacetate (5.34 g; 46.8 mmol), $SnCl_2$ (85 mg; 4.5 mmol) and cyclohexanecarboxaldehyde (5.0 g; 45 mmol) in CH_2Cl_2 (20 ml). The crude oily residue was purified by fractional distillation under reduced pressure to give **50** as a pale yellow oil (5.38 g; 66%). R_f: 0.63 ($CHCl_3$); $bp_{0.3}$ 135–139°C. ([412]Lit. bp_{18}: 146-150°C); MS (FAB$^+$) *m/z*: 199.0 [100, (M+H)$^+$]; MS (FAB$^-$) *m/z*: 197.0 [100, (M-H)$^-$]; Acc. MS (FAB$^+$): 199.1341, $C_{11}H_{19}O_3$ requires 199.1334; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 1.28 (t, 3H, CH$_2$C<u>H</u>$_3$, J= 7.2 Hz), 1.39-2.49 (m, 11H, cyclohexyl H), 3.48 (s, 2H, 2-CH$_2$) and 4.19 (q, 2H, C<u>H</u>$_2$CH$_3$, J= 7.2 Hz).

8.4.21 4-Cyclohexyl-7-hydroxycoumarin (51)

Prepared by method 8.2.4, using resorcinol (1.67 g; 15.1 mmol), **50** (3.0 g; 15 mmol) and a mixture of CF_3COOH (2.33 ml; 30.3 mmol) and conc. H_2SO_4 (1.5 ml; 30 mmol). The crude yellow solid was purified flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from hot acetone to give **51** as white crystals (1.45 g; 39%). R_f: 0.80 ($CHCl_3$/acetone, 4:1); mp 191-192°C ([230]Lit mp 176–178°C); MS (FAB^+) *m/z*: 489.1 [15, $(2M+H)^+$], 245.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 487.3 [15, $(2M-H)^-$], 243.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 245.1179, $C_{15}H_{17}O_3$ requires 245.1178; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.38-1.87 (m, 11H, cyclohexyl H), 6.04 (s, 1H, C_3-H), 6.71 (d, 1H, C_8-H, J = 2.3 Hz), 6.81 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.70 (d, 1H, C_5-H, J = 8.8 Hz) and 10.56 (s, 1H, OH); Found C, 73.90; H, 6.60; $C_{15}H_{16}O_3$ requires C, 73.75; H, 6.60.

8.4.22 4-Cyclohexylcoumarin-7-*O*-sulphamate (52)

Compound **51** (400 mg; 1.6 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **52** as white fine crystals (192 mg; 36%). R_f: 0.45 ($CHCl_3$/ethyl acetate, 4:1); mp 187–190°C; MS (FAB^+) *m/z*: 324.1 [100, $(M+H)^+$], 245.1 [15, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 323.2 [100, $(M-H)^-$], 243.2 [50, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 324.0902, $C_{15}H_{18}NO_5S$ requires 324.0906; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.23-2.06 (m, 11H, cyclohexyl H), 6.32 (s, 1H, C_3-H), 7.32 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.36 (d, 1H, C_8-H, J = 2.3 Hz), 8.01 (d, 1H, C_5-H, J = 8.9 Hz) and 8.31 (s, 2H, NH_2); Found C, 55.70; H, 5.28; N, 4.17; $C_{15}H_{17}NO_5S$ requires C, 55.72; H, 5.30; N, 4.33%.

8.4.23 (Adamantan-1-yl)acetyl chloride (53)

1-Adamantaneacetic acid (5.0 g; 25.7 mmol) in an excess of thionyl chloride (20 ml; 77.2 mmol) and THF (5 ml) was boiled under reflux overnight under N_2. Thionyl chloride was removed under vacuum to get the crude **53** as a brown oil (5.5 g; 101%

crude), which was used for the next reaction without purification. R_f: 0.91 ($CHCl_3$/MeOH, 8:1); ([417]Lit. bp_3 107-109°C); MS (FAB^+) *m/z*: 213.1 [100, $(M(^{35}Cl)+H)^+$]; MS (FAB^-) *m/z*: 211.1 [100, $(M(^{35}Cl)-H)^-$]; Acc. MS *m/z* (FAB^+): 213.1097, $C_{12}H_{18}{}^{35}ClO$ requires 213.1081 and 215.1006, $C_{12}H_{18}{}^{37}ClO$ requires 215.1012; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.11-1.76 (m, 15H, adamantane H) and 2.69 (s, 2H, CH_2CO).

8.4.24 4-(Adamantan-1-yl)-3-oxobutanoate (54)

Prepared by method A (8.2.1), using ethyl potassium malonate (8.4 g; 49 mmol), CH_3CN (150 ml), Et_3N (11 ml; 75 mmol), $MgCl_2$ (5.6 g; 59 mmol) and **53** (5.0 g; 24 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **54** as brown oil (4.5 g; 72%). R_f: 0.77 ($CHCl_3$/Hexane, 9:1); ([418]Lit. $bp_{0.07}$ 105-106°C); MS (FAB^+) *m/z*: 265.2 [100, $(M+H)^+$], 135 [85, $(C_{10}H_{15}+H)^+$]; MS (FAB^-) *m/z*: 263.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 265.1697, $C_{16}H_{25}O_3$ requires 265.1705; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.24-1.58 (m, 15H, adamantane H), 1.28 (t, 3H, $CH_2C\underline{H}_3$), 2.28 (s, 2H, CH_2CO), 3.41 (s, 2H, 2-CH_2) and 4.21 (q, 2H, $C\underline{H}_2CH_3$, J= 7.0 Hz),

8.4.25 4-(1-Adamantanemethyl)-7-hydroxycoumarin (55)

Prepared by method 8.2.4, using resorcinol (834 mg; 7.6 mmol), **54** (2.0 g; 7.6 mmol) and a mixture of CF_3COOH (1.2 ml; 15 mmol) and conc. H_2SO_4 (1.5 ml; 15 mmol). The crude yellow solid was purified by recrystallisation from THF:hexane to give **55** as yellow crystals (1.82 g; 77%). R_f: 0.72 ($CHCl_3$/acetone, 4:1); mp 211-214°C; MS (FAB^+) *m/z*: 311.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 309.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 311.1568, $C_{20}H_{23}O_3$ requires 311.1569); ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.52-2.09 (m, 15H, adamantane H), 1.90 (s, 2H, CH_2), 5.95 (s, 1H, C_3-H), 6.69 (d, 1H, C_8-H, J = 2.3 Hz), 6.78 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.75 (d, 1H, C_5-H, J = 8.9 Hz) and 10.54 (s, 1H, OH); Found C, 77.42; H, 7.15; $C_{20}H_{22}O_3$ requires C, 77.39; H, 7.14%.

8.4.26 4-(1-Adamantanemethyl)coumarin-7-*O*-sulphamate (56)

Compound **55** (400 mg; 1.3 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **56** as white fine crystals (88 mg; 18%). R_f: 0.57 ($CHCl_3$/ethyl acetate, 4:1); mp: 218-221°C; MS (FAB^+) *m/z*: 390.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 388.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 390.1301, $C_{20}H_{24}NO_5S$ requires 390.1297; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.54-1.64 (m, 15H, adamantane H), 1.91 (s, 2H, CH_2), 6.24 (s, 1H, C_3-H), 7.24-7.31 (m, 2H, C_6-H and C_8-H), 8.04 (d, 1H, C_5-H, J = 8.9 Hz) and 8.25 (s, 2H, NH_2); Found C, 61.40; H, 5.75; N, 3.22; $C_{20}H_{23}NO_5S$ requires C, 61.68; H, 5.95; N, 3.60%.

8.5 Synthesis of compounds with a methyl group at the C-4 position and varying lengths of alkyl chain at the C-3 position of the coumarin ring

8.5.1 Ethyl 2-acetylheptanoate (57)

Prepared by method 8.2.2, using K_2CO_3 (11.09 g; 79.2 mmol), water (50 ml), 1-bromopentane (4.09 ml; 33 mmol), ethyl 3-oxobutanoate (4.29 ml; 33 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 33 mmol). The crude oily residue (4.68 g) was purified by flash chromatography ($CHCl_3$) to give **57** as a pale yellow oil. (4.03 g; 61%). ([240]Lit. bp_8 101-105°C); R_f: 0.69 ($CHCl_3$); MS (FAB^+) *m/z*: 201.2 [100, $(M+H)^+$]; Acc. MS (FAB^+): 201.1492, $C_{11}H_{21}O_3$ requires 201.1491; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, CH_3, J = 7.3 Hz), 1.19 (t, 3H, $CH_2C\underline{H}_3$, J = 7.3 Hz), 1.23-1.27 (m, 6H, 3×CH_2), 1.68-1.72 (q, 2H, 3-CH_2, J = 6.1 Hz), 2.17 (s, 3H, CH_3CO), 3.57 (t, 1H, 2-H, J = 7.3 Hz) and 4.12 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.5.2 7-Hydroxy-4-methyl-3-pentylcoumarin (58)

Prepared by method 8.2.4, using resorcinol (2.2 g; 20 mmol), **57** (4.0 g; 20 mmol) and a mixture of CF_3COOH (3.08 ml; 40 mmol) and conc. H_2SO_4 (2.04 ml; 40 mmol). The crude pale yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to

4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **58** as white crystals (2.03 g; 38%). R_f: 0.80 ($CHCl_3$/acetone, 3:1); mp 101–102°C ([401]Lit. mp 111-113°C); MS (FAB^+) *m/z*: 493.4 [15, $(2M+H)^+$], 247.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 491.3 [10, $(2M-H)^-$], 245.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 247.1327, $C_{15}H_{19}O_3$ requires 247.1334; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.87 (t, 3H, 5'-CH_3, J = 6.8 Hz), 1.29-1.44 (m, 6H, 3×CH_2), 2.35 (s, 3H, C_4-CH_3), 2.51 (t, 2H, 1'-CH_2, J = 7.3 Hz), 6.71 (d, 1H, C_8-H, J = 2.4 Hz), 6.78 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.59 (d, 1H, C_5-H, J = 8.8 Hz) and 10.36 (s, 1H, OH); Found C, 72.90; H, 7.29; $C_{15}H_{18}O_3$ requires C, 73.15; H, 7.37%.

8.5.3 4-Methyl-3-pentylcoumarin-7-*O*-sulphamate (59)

Compound **58** (700 mg; 2.84 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **59** as white fine crystals (479 mg; 52%). R_f: 0.82 ($CHCl_3$/ethyl acetate, 4:1); mp 133–135°C; MS (FAB^+) *m/z*: 326.2 [100, $(M+H)^+$], 245.2 [50, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 326.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): Acc. MS (FAB^+): 326.1076, $C_{15}H_{20}NO_5S$ requires 326.1062; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.87 (t, 3H, 5'-CH_3, J = 6.7 Hz), 1.31-1.49 (m, 6H, 3×CH_2), 2.43 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 8.2 Hz), 7.26-7.29 (m, 2H, C_6-H and C_8-H), 7.88 (d, 1H, C_5-H, J = 7.9 Hz) and 8.19 (s, 2H, NH_2); Found C, 55.20; H, 5.88; N, 4.27; $C_{15}H_{19}NO_5S$ requires C, 55.37; H, 5.89; N, 4.30%.

8.5.4 Ethyl 2-acetyloctanoate (60)

Prepared by method 8.2.2, using K_2CO_3 (11.1 g; 79.2 mmol), water (50 ml), 1-bromohexane (4.63 ml; 33 mmol), ethyl 3-oxobutanoate (4.29 ml; 33 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 33 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **60** as a pale yellow oil (3.05 g; 43%). ([241]Lit. bp_{10} 127-129°C); R_f: 0.66 ($CHCl_3$); MS (FAB^+) *m/z*: 215.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*:

213.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 215.1600, $C_{12}H_{23}O_3$ requires 215.1639; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, CH_3, J = 6.7 Hz), 1.18 (t, 3H, $CH_2C\underline{H}_3$, J = 7.1 Hz), 1.22-1.36 (m, 8H, 4×CH_2), 1.71 (q, 2H, 3-CH_2, J = 6.4 Hz), 2.17 (s, 3H, CH_3CO), 3.57 (t, 1H, 2-H, J = 6.7 Hz) and 4.12 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.5.5 3-Hexyl-7-hydroxy-4-methylcoumarin (61)

Prepared by method 8.2.4, using resorcinol (1.28 g; 11.7 mmol), **60** (2.5 g; 12 mmol) and a mixture of CF_3COOH (1.8 ml; 23 mmol) and conc. H_2SO_4 (1.19 ml; 23.3 mmol). The crude pale white solid was recrystallised from acetone/hexane to give **61** as white fine crystals (2.12 g; 70%). R_f: 0.81 ($CHCl_3$/acetone, 3:1); mp 112–114°C ([401]Lit. mp 111-112°C); MS (FAB^+) *m/z*: 521.1 [100, $(2M+H)^+$], 261.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 259.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 261.1421, $C_{16}H_{21}O_3$ requires 261.1412; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88 (t, 3H, 6'-CH_3, J = 7.1 Hz), 1.25-1.55 (m, 8H, 4×CH_2), 2.39 (s, 3H, C_4-CH_3), 2.63 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.83 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 6.96 (d, 1H, C_8-H, J = 2.4 Hz), 7.49 (d, 1H, C_5-H, J = 8.8 Hz) and 10.41 (s, 1H, OH); Found C, 73.90; H, 7.78; $C_{16}H_{20}O_3$ requires C, 73.82; H, 7.74%.

8.5.6 3-Hexyl-4-methylcoumarin-7-*O*-sulphamate (62)

Compound **61** (700 mg; 2.69 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid that isolated was recrystallised from ethyl acetate/hexane to give **62** as white fine fluffy crystals (449 mg; 49%). R_f: 0.41 ($CHCl_3$/ethyl acetate, 4:1); mp 132–133°C; MS (FAB^+) *m/z*: 679.4 [15, $(2M+H)^+$], 340.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 338.2 [100, $(M-H)^-$], 259.2 [45, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 340.1230, $C_{16}H_{22}NO_5S$ requires 340.1219; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, CH_3, J = 5.5 Hz), 1.25-1.48 (m, 8H, 4×CH_2), 2.43 (s, 3H, C_4-H_3), 2.59 (t, 2H, 1'-CH_2, J = 7.1 Hz), 7.26 (d, 1H, C_8-H, J = 2.1 Hz), 7.28 (m, 1H, C_6-H), 7.88 (d, 1H, C_5-H, J = 7.3 Hz) and 8.19 (s, 2H, NH_2); Found C, 56.90; H, 6.22; N, 4.12; $C_{16}H_{21}NO_5S$ requires C, 56.62; H, 6.24; N, 4.13%.

8.5.7 Ethyl 2-acetylnonanoate (63)

Prepared by method 8.2.2, using K_2CO_3 (11.11 g; 80.39 mmol), water (50 ml), 1-bromoheptane (6 ml; 34 mmol), ethyl acetoacetate (4.27 ml; 33.5 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 34 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **63** as a pale yellow oil (2.45 g; 33%). ([419]Lit. $bp_{0.4}$ 80-85°C); R_f: 0.9 ($CHCl_3$); MS (FAB^+) *m/z*: 229.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 227.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 229.1804, $C_{13}H_{25}O_3$ requires 229.1803; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.85 (t, 3H, CH_3, J = 7.1 Hz), 1.18 (t, 3H, $CH_2C\underline{H}_3$ J = 7.1 Hz), 1.23-1.73 (m, 12H, 6×CH_2), 2.17 (s, 3H, CH_3CO), 3.56 (t, 1H, 2-H, J = 7.1 Hz) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.1 Hz).

8.5.8 3-Heptyl-7-hydroxy-4-methylcoumarin (64)

Prepared by method 8.2.4, using resorcinol (965 mg; 8.76 mmol), **63** (2.0 g; 8.8 mmol) and a mixture of CF_3COOH (1.55 ml; 20.2 mmol) and conc. H_2SO_4 (1.03 ml; 20.2 mmol). The crude pale yellow solid was recrystallised from acetone/hexane to give **64** as pale yellow crystals (730 mg; 30%). R_f: 0.78 ($CHCl_3$/acetone, 3:1); mp 96–98°C; MS (FAB^+) *m/z*: 549.4 [15, $(2M+H)^+$], 275.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 547.4 [10, $(2M-H)^-$], 273.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 275.1641, $C_{17}H_{23}O_3$ requires 275.1647; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.3 Hz), 1.26-1.53 (m, 10H, 5×CH_2), 2.39 (s, 3H, C_4-CH_3), 2.63 (t, 2H, 1'-CH_2), 7.01 (d, 1H, C_8-H, J = 2.4 Hz), 6.85 (dd, 1H, C_6-H, J = 2.4, 8.7 Hz), 7.49 (d, 1H, C_5-H, J = 8.85 Hz) and 10.37 (s, 1H, OH); Found C, 74.08; H, 8.03; $C_{17}H_{22}O_3$ requires C, 74.42; H, 8.08%.

8.5.9 3-Heptyl-4-methylcoumarin-7-*O*-sulphamate (65)

Compound **64** (400 mg; 1.46 mmol) was sulphamoylated by method 8.2.5. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **65** as white fine crystals (75 mg; 14%). R_f: 0.51 ($CHCl_3$/ethyl acetate 4:1); mp 138–140°C; MS (FAB^+) *m/z*: 707.2 [40, $(2M+H)^+$], 354.1 [100, $(M+H)^+$]; MS (FAB^-)

m/z: 705.2 [40, $(2M-H)^-$], 352.1 [100, $(M-H)^-$], 273.2 [90, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 354.1391, $C_{17}H_{24}NO_5S$ requires 354.1375; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, CH_3, J = 6.7 Hz), 1.26-1.49 (m, 10H, 5×CH_2), 2.43 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 7.1 Hz), 7.25-7.29 (m, 2H, C_6-H and C_8-H), 7.88 (d, 1H, C_5-H, J = 8.8 Hz) and 8.18 (s, 2H, NH_2); Found C, 57.80; H, 6.58; N, 3.92; $C_{17}H_{23}NO_5S$ requires C, 57.77; H, 6.56; N, 3.96%.

8.5.10 Ethyl 2-acetyldecanoate (66)

Prepared by method 8.2.2, using K_2CO_3 (11.47 g; 82.98 mmol), water (50 ml), 1-bromooctane (6.0 ml; 35 mmol), ethyl 3-oxobutanoate (4.41 ml; 34.6 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 35 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$/acetone) to give **66** as a pale yellow oil (3.1 g; 37%). ([420]Lit. $bp_{0.4}$ 79-83°C); R_f: 0.68 ($CHCl_3$); MS (FAB^+) *m/z*: 243.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 241.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 243.1968, $C_{14}H_{27}O_3$ requires 243.1960; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, CH_3, J = 6.7 Hz), 1.18 (t, 3H, $CH_2C\underline{H}_3$, J = 7.3 Hz), 1.23-1.75 (m, 12H, 6×CH_2), 2.06 (s, 3H, CH_3CO), 2.39 (q, 2H, 3-CH_2, J = 7.3 Hz), 3.61 (t, 1H, 2-H, J = 6.7 Hz) and 4.12 (q, 2H, $C\underline{H}_2CH_3$, J = 7.1 Hz).

8.5.11 7-Hydroxy-4-methyl-3-octylcoumarin (67)

Prepared by method 8.2.4, using resorcinol (910 mg; 8.26 mmol), **66** (2.0 g; 8.3 mmol) and a mixture of CF_3COOH (1.27 ml; 16.3 mmol) and conc. H_2SO_4 (0.84 ml; 16.3 mmol). The crude brown solid was recrystallised from acetone/hexane to give **67** as white crystals (405 mg; 17%). R_f: 0.72 ($CHCl_3$/acetone, 3:1); mp 98–100°C; MS (FAB^+) *m/z*: 577.4 [40, $(2M+H)^+$], 289.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 575.3 [35, $(2M-H)^-$], 287.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 289.1801, $C_{18}H_{25}O_3$ requires 289.1801; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88 (t, 3H, CH_3, J = 7.3 Hz), 1.27-1.46 (m, 12H, 6×CH_2), 2.38 (s, 3H, C_4-CH_3), 2.54 (t, 2H, 1'-CH_2, J = 6.4 Hz), 6.69 (d, 1H, C_8-H, J = 2.1 Hz), 6.81 (dd, 1H, C_6-H, J = 2.1, 8.8 Hz), 7.62 (d, 1H, C_5-H, J = 8.8 Hz)

and 10.52 (s, 1H, OH); Found C, 74.90; H, 8.40; $C_{18}H_{24}O_3$ requires C, 74.97; H, 8.39%.

8.5.12 4-Methyl-3-octylcoumarin-7-*O*-sulphamate (68)

Compound **67** (400 mg; 1.39 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **68** as white fine crystals (69 mg; 15%). R_f: 0.43 ($CHCl_3$/ethyl acetate, 4:1); mp 135–138°C; MS (FAB^+) *m/z*: 735.2 [35, $(2M+H)^+$], 368.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 733.3 [40, $(2M-H)^-$], 366.2 [100, $(M-H)^-$], 287.2 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 368.1529, $C_{18}H_{26}NO_5S$ requires 368.1532; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.86 (t, 3H, CH_3, J = 7.3 Hz), 1.25-1.49 (m, 12H, 6×CH_2), 2.43 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 7.1 Hz), 7.26-7.29 (m, 2H, C_6-H and C_8-H), 7.88 (d, 1H, C_5-H, J = 8.8 Hz) and 8.18 (s, 2H, NH_2); Found C, 58.70; H, 6.72; N, 3.68; $C_{18}H_{25}NO_5S$ requires C, 58.84; H, 6.86; N, 3.81%.

8.5.13 Ethyl 2-acetylundecanoate (69)

Prepared by method 8.2.2, using K_2CO_3 (7.4 g; 58 mmol), water (50 ml), 1-bromononane (5.0 ml; 24 mmol), ethyl 3-oxobutanoate (3.1 ml; 24 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 49 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **69** as a pale yellow oil (2.76 g; 45%). ([421]Lit. $bp_{4.2}$ 191°C); R_f: 0.65 ($CHCl_3$); MS (FAB^+) *m/z*: 257.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 255.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 257.2126, $C_{15}H_{29}O_3$ requires 257.2170; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 6.8 Hz), 1.26-1.91 (m, 19H, $CH_2C\underline{H}_3$ and 8×CH_2), 2.22 (s, 3H, CH_3CO), 3.39 (t, 1H, 2-H, J = 7.3 Hz) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.5.14 7-Hydroxy-4-methyl-3-nonylcoumarin (70)

Prepared by method 8.2.4, using resorcinol (1.07 g; 9.76 mmol), **69** (2.5 g; 9.8 mmol) and a mixture of CF_3COOH (1.5 ml; 20 mmol) and conc. H_2SO_4 (1.0 ml; 20 mmol). The crude yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **70** as white crystals (797 mg; 27%). R_f: 0.69 ($CHCl_3$/acetone, 3:1); mp 78–80°C; MS (FAB^+) *m/z*: 605.3 [35, $(2M+H)^+$], 303.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 603.1 [40, $(2M-H)^-$], 301.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 303.1964, $C_{19}H_{27}O_3$ requires 303.1960; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.87 (t, 3H, CH_3, J = 7.1 Hz), 1.26-1.55 (m, 14H, 7×CH_2), 2.39 (s, 3H, C_4-CH_3), 2.63 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.86 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.05 (d, 1H, C_8-H, J = 2.4 Hz), 7.49 (d, 1H, C_5-H, J = 8.8 Hz) and 7.55 (s, 1H, OH); Found C, 75.35; H, 8.65; $C_{19}H_{26}O_3$ requires C, 75.46; H, 8.67%.

8.5.15 4-Methyl-3-nonylcoumarin-7-*O*-sulphamate (71)

Compound **70** (400 mg; 1.32 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **71** as white fine crystals (184 mg; 37%). R_f: 0.82 ($CHCl_3$/ethyl acetate, 4:1); mp 125–129°C; MS (FAB^+) *m/z*: 763.3 [45, $(2M+H)^+$], 382.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 761.2 [45, $(2M-H)^-$], 380.1 [100, $(M-H)^-$], 301.1 [75, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 382.1679, $C_{19}H_{28}NO_5S$ requires 382.1688; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, CH_3, J = 6.7 Hz), 1.24-1.48 (m, 14H, 7×CH_2), 2.42 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 7.3 Hz), 7.25 (d, 1H, C_8-H, J = 2.4 Hz), 7.28 (dd, 1H, C_6-H, J = 2.1, 8.5 Hz), 7.88 (d, 1H, C_5-H, J = 8.5 Hz) and 8.19 (s, 2H, NH_2); Found C, 59.50; H, 7.08; N, 3.59; $C_{19}H_{27}NO_5S$ requires C, 59.82; H, 7.13; N, 3.67%.

8.5.16 Ethyl 2-acetyldodecanoate (72)

Prepared by method 8.2.2, using K_2CO_3 (7.5 g; 54 mmol), water (50 ml), 1-bromodecane (5.0 ml; 23 mmol), ethyl 3-oxobutanoate (2.88 ml; 22.6 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 45 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **72** as a pale yellow oil (2.95 g; 48%). ([422]Lit. bp_2 140-150°C); R_f: 0.76 ($CHCl_3$); MS (FAB^+) *m/z*: 271.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 269.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 271.2275, $C_{16}H_{31}O_3$ requires 271.2273; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.3 Hz), 1.25-2.00 (m, 21H, $CH_2C\underline{H}_3$ and 9×CH_2), 2.22 (s, 3H, CH_3CO), 3.39 (t, 1H, 2-H, J = 7.3 Hz) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.5.17 7-Hydroxy-3-decyl-4-methylcoumarin (73)

Prepared by method 8.2.4, using resorcinol (1.02 g; 9.25 mmol), **72** (2.5g; 9.3 mmol) and a mixture of CF_3COOH (1.42 ml; 18.5 mmol) and conc. H_2SO_4 (0.94 ml; 18.5 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **73** as white crystals (807 mg; 28%). R_f: 0.81 ($CHCl_3$/acetone, 3:1); mp 96–100°C; MS (FAB^+) *m/z*: 633.3 [50, $(2M+H)^+$], 317.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 631.2 [10, $(2M-H)^-$], 315.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 317.2117, $C_{20}H_{29}O_3$ requires 317.2117; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.3 Hz), 1.26-1.55 (m, 16H, 8×CH_2), 2.39 (s, 3H, C_4-CH_3), 2.63 (t, 2H, 1'-CH_2, J = 7.6 Hz), 6.85 (dd, 1H, C_6-H, J = 2.4, 8.7 Hz), 7.02 (d, 1H, C_8-H, J = 2.4 Hz), 7.32 (s, 1H, OH) and 7.49 (d, 1H, C_5-H, J = 8.5 Hz); Found C, 75.65; H, 8.99 $C_{20}H_{28}O_3$ requires C, 75.91; H, 8.92%.

8.5.18 3-Decyl-4-methylcoumarin-7-*O*-sulphamate (74)

Compound **73** (400 mg; 1.27 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane

to give **74** as white fine crystals (101 mg; 27%). R_f: 0.55 ($CHCl_3$/ethyl acetate, 4:1); mp 118–121°C; MS (FAB^+) *m/z*: 791.3 [20, $(2M+H)^+$], 396.1 [100, $(M+H)^+$], 317.1 [30, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 394.1 [100, $(M-H)^-$], 315.1 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 396.1850, $C_{20}H_{30}NO_5S$ requires 396.1845; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, CH_3, J = 7.6 Hz), 1.24-1.47 (m, 16H, 8×CH_2), 2.43 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 7.3 Hz), 7.25 (d, 1H, C_8-H, J = 2.4 Hz), 7.27 (m, 1H, C_6-H); 7.87 (d, 1H, C_5-H, J = 8.5 Hz) and 8.13 (s, 2H, NH_2); Found C, 60.65; H, 7.42; N, 3.12 $C_{20}H_{29}NO_5S$ requires C, 60.74; H, 7.39; N, 3.54%.

8.5.19 Ethyl 2-acetyltridecanoate (75)

Prepared by method 8.2.2, using K_2CO_3 (7.1 g; 51 mmol), water (50 ml), 1-bromoundecane (5.0 ml; 21 mmol), ethyl 3-oxobutanoate (2.71 ml; 21.3 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 43 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$/hexane, 8:1 to 2:1 gradient) to give **75** as a pale yellow oil (3.25 g; 54%). R_f: 0.69 ($CHCl_3$/hexane, 2:1); ([423]Lit. bp_1 145-150°C); MS (FAB^+) *m/z*: 285.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 283.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 285.2422, $C_{17}H_{33}O_3$ requires 285.2429; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.3 Hz), 1.25-1.85 (m, 23H, $CH_2C\underline{H}_3$ and 10×CH_2), 2.22 (s, 3H, CH_3CO), 3.39 (t, 1H, 2-H, J = 7.3 Hz) and 4.15 (q, 2H, $C\underline{H}_2CH_3$, J = 7.3 Hz).

8.5.20 7-Hydroxy-4-methyl-3-undecylcoumarin (76)

Prepared by method 8.2.4, using resorcinol (968 mg; 8.8 mmol) and **75** (2.5 g; 8.8 mmol) in the presence of CF_3COOH (1.4 ml; 18 mmol) and conc. H_2SO_4 (0.9 ml; 18 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale brown solid isolated was recrystallised from acetone/hexane to give **76** as white crystals (722 mg; 29%). R_f: 0.88 ($CHCl_3$/acetone, 3:1); mp 74–76°C; MS (FAB^+) *m/z*: 660.9 [35, $(2M+H)^+$], 331.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 659.0 [10, $(2M-H)^-$], 329.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 331.2268, $C_{21}H_{31}O_3$ requires 331.2273; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.85 (t, 3H, CH_3, J = 7.0

Hz), 1.23-1.41 (m, 18H, 9×CH_2), 2.35 (s, 3H, C_4-CH_3), 2.51 (t, 2H, 1'-CH_2, J = 7.4 Hz), 6.67 (d, 1H, C_8-H, J = 2.3 Hz), 6.78 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.59 (d, 1H, C_5-H, J = 8.9 Hz) and 10.39 (s, 1H, OH); Found C, 76.10; H, 8.96 $C_{21}H_{30}O_3$ requires C, 76.33; H, 9.15%.

8.5.21 4-Methyl-3-undecylcoumarin-7-*O*-sulphamate (77)

Compound **76** (200 mg; 0.71 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **77** as white fine crystals (11 mg; 4%). R_f: 0.49 ($CHCl_3$/ethyl acetate, 4:1); mp 117-119°C; MS (FAB$^+$) *m/z*: 410.3 [100, $(M+H)^+$]; MS (FAB$^-$) *m/z*: 408.3 [100, $(M-H)^-$]; Acc. MS (FAB$^+$): 410.1992, $C_{21}H_{32}NO_5S$ requires 410.2001; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 0.87 (t, 3H, CH_3, J = 7.2 Hz), 1.22-1.45 (m, 18H, 9×CH_2), 2.34 (s, 3H, C_4-CH_3), 2.59 (t, 2H, 1'-CH_2, J = 7.3 Hz), 6.89 (d, 1H, C_8-H, J = 2.3 Hz), 7.19 (dd, 1H, C_6-H, J = 2.3, 8.8 Hz); 7.59 (d, 1H, C_5-H, J = 8.9 Hz) and 8.18 (s, 2H, NH_2); Found C, 61.40; H, 7.75; N, 3.16, $C_{21}H_{31}NO_5S$ requires C, 61.59; H, 7.63; N, 3.42%.

8.5.22 Ethyl 2-acetyltetradecanoate (78)

Prepared by method 8.2.2, using K_2CO_3 (7.99 g; 57.8 mmol), water (60 ml), 1-bromododecane (6.0 ml; 24 mmol), ethyl 3-oxobutanoate (3.1 ml; 24 mmol), CH_2Cl_2 (60 ml) and Bu_4NCl (~10 g; 48 mmol). The crude oily residue was purified by distillation under reduced pressure to give **78** as a pale yellow oil (3.65 g; 51%). R_f: 0.66 (CH_2Cl_2); $bp_{0.3}$ 159-160°C. ([424]Lit. $bp_{1.1}$ 149-152°C); MS (FAB$^+$) *m/z*: 299.2 [100, $(M+H)^+$]; MS (FAB$^-$) *m/z*: 297.2 [100, $(M-H)^-$]; Acc. MS (FAB$^+$): 299.2594, $C_{18}H_{35}O_3$ requires 299.2586; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.12-1.89 (m, 25H, $CH_2C\underline{H}_3$ and 11×CH_2), 2.22 (s, 3H, CH_3CO), 3.53 (t, 1H, 2-H, J = 7.0 Hz) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz).

8.5.23 3-Dodecyl-7-hydroxy-4-methylcoumarin (79)

Prepared by method 8.2.4, using resorcinol (553 mg; 5.02 mmol), **78** (1.5 g; 5.0 mmol) and a mixture of CF_3COOH (0.8 ml; 10 mmol) and conc. H_2SO_4 (0.6 ml; 10 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **79** as off-white crystals (146 mg; 9%). R_f: 0.74 ($CHCl_3$/acetone, 3:1); mp 94-96°C; MS (FAB^+) *m/z*: 689.4 [20, $(2M+H)^+$], 345.4 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 343.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 345.2435, $C_{22}H_{33}O_3$ requires 345.2429; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.25-1.63 (m, 20H, 10×CH_2), 2.38 (s, 3H, C_4-CH_3), 2.63 (t, 2H, 1'-CH_2, J = 7.4 Hz), 6.19 (s, 1H,), 6.81 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 6.91 (d, 1H, C_8-H, J = 2.3 Hz) and 7.48 (d, 1H, C_5-H, J = 8.9 Hz); Found C, 76.60; H, 9.22; $C_{22}H_{32}O_3$ requires C, 76.70; H, 9.36%.

8.5.24 3-Dodecyl-4-methylcoumarin-7-*O*-sulphamate (80)

Compound **79** (100 mg; 0.29 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by preparative TLC ($CHCl_3$/ethyl acetate, 6:1) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **80** as white fine crystals (15 mg; 12%). R_f: 0.36 ($CHCl_3$/ethyl acetate, 6:1); mp 157-159°C; MS (FAB^+) *m/z*: 847.1 [15, $(2M+H)^+$], 424.1 [100, $(M+H)^+$], 245.1 [30, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 422.1 [100, $(M-H)^-$], 343.2 [55, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 424.1246, $C_{22}H_{34}NO_5S$ requires 424.1241; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88 (t, 3H, CH_3, J = 7.2 Hz), 1.24-1.48 (m, 20H, 10×CH_2), 2.45 (s, 3H, C_4-CH_3), 2.61 (t, 2H, 1'-CH_2, J = 7.4 Hz), 7.23 (d, 1H, C_8-H, J = 2.3 Hz), 7.27 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz); 7.89 (d, 1H, C_5-H, J = 8.9 Hz) and 8.21 (s, 2H, NH_2).

8.5.25 Ethyl 2-acetylpentadecanoate (81)

Prepared by method 8.2.2, using K_2CO_3 (6.3 g; 46 mmol), water (60 ml), 1-bromotridecane (5.0 ml; 19 mmol), ethyl 3-oxobutanoate (2.42 ml; 18.9 mmol), CH_2Cl_2 (60 ml) and Bu_4NCl (11.0 g; 37.9 mmol). The crude oily residue was purified by distillation under reduced pressure to give **81** as a pale yellow oil (2.88 g; 49%). R_f:

0.77 ($CHCl_3$); $bp_{0.3}$ 153-155°C; MS (FAB^+) *m/z*: 313.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 311.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 313.2756, $C_{19}H_{37}O_3$ requires 313.2743; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.12-1.56 (m, 27H, $CH_2C\underline{H}_3$ and 12×CH_2), 2.22 (s, 3H, CH_3CO), 3.53 (t, 1H, 2-H, J = 6.7 Hz) and 4.21 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz).

8.5.26 7-Hydroxy-4-methyl-3-tridecylcoumarin (82)

Prepared by method 8.2.4, using resorcinol (705 mg; 6.4 mmol), **81** (2.0 g; 6.4 mmol) and a mixture of CF_3COOH (1.0 ml; 13 mmol) and conc. H_2SO_4 (0.7 ml; 13 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **82** as white crystals (621 mg; 27%). R_f: 0.65 ($CHCl_3$/acetone, 3:1); mp 71-72°C; MS (FAB^+) *m/z*: 359.4 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 357.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 359.2599, $C_{23}H_{35}O_3$ requires 359.2586; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.26-1.84 (m, 22H, 11×CH_2), 2.38 (s, 3H, C_4-CH_3), 2.62 (t, 2H, 1'-CH_2, J = 7.4 Hz), 5.87 (s, 1H, OH), 6.81 (dd, 1H, C_6-H, J = 2.3, 8.7 Hz), 6.86 (d, 1H, C_8-H, J = 2.3 Hz) and 7.48 (d, 1H, C_5-H, J = 8.9 Hz); Found C, 77.20; H, 10.00; $C_{23}H_{34}O_3$ requires C, 77.05; H, 9.56%.

8.5.27 4-Methyl-3-tridecylcoumarin-7-*O*-sulphamate (83)

Compound **82** (400 mg; 1.12 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **83** as white fine crystals (35 mg; 7%). R_f: 0.67 ($CHCl_3$/ethyl acetate, 6:1); mp 115-119°C; MS (FAB^+) *m/z*: 438.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 436.2 [100, $(M-H)^-$], 357.2 [30, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 438.2291, $C_{23}H_{36}NO_5S$ requires 438.2272; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.84 (t, 3H, CH_3, J = 7.0 Hz), 1.23-1.44 (m, 22H, 11×CH_2), 2.42 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 7.8 Hz), 7.26 (d, 1H, C_8-H, J = 2.3 Hz), 7.28 (m, 1H, C_6-H), 7.88 (d, 1H, C_5-H, J = 8.6 Hz) and 8.21 (s, 2H,

NH_2); Found C, 63.60; H, 7.98; N, 3.17; $C_{23}H_{35}NO_5S$ requires C, 63.13; H, 8.06; N, 3.20%.

8.5.28 Ethyl 2-acetylhexadecanoate (84)

Prepared by method 8.2.2, using K_2CO_3 (5.6 g; 43 mmol), water (60 ml), 1-bromotetradecane (5.0 ml; 18 mmol), ethyl 3-oxobutanoate (2.3 ml; 18 mmol), CH_2Cl_2 (60 ml) and Bu_4NCl (11.0 g; 36.1 mmol). The crude oily residue was purified by distillation under reduced pressure to give **84** as a pale yellow oil (2.32 g; 23%). R_f: 0.65 ($CHCl_3$); $bp_{0.3}$ 158-162°C. ([425]Lit. $bp_{0.5}$ 162 °C); MS (FAB^+) *m/z*: 327.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 325.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 327.2900, $C_{20}H_{39}O_3$ requires 327.2899; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.20-1.81 (m, 29H, $CH_2C\underline{H}_3$ and 13×CH_2), 2.17 (s, 3H, CH_3CO), 3.53 (t, 1H, 2-H, J = 7.0 Hz) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz).

8.5.29 7-Hydroxy-4-methyl-3-tetradecylcoumarin (85)

Prepared by method 8.2.4, using resorcinol (337 mg; 3.1 mmol), **84** (1.0 g; 3.1 mmol) and a mixture of CF_3COOH (0.5 ml; 6.2 mmol) and conc. H_2SO_4 (0.4 ml; 6.2 mmol). The crude brown residue was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) to give **85** as pale yellow waxy solid (472 mg; 41%). R_f: 0.74 ($CHCl_3$/acetone, 3:1); mp 64-66°C; MS (FAB^+) *m/z*: 373.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 371.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 373.2754, $C_{24}H_{37}O_3$ requires 373.2743; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.12-1.82 (m, 24H, 12×CH_2), 2.38 (s, 3H, C_4-CH_3), 2.62 (t, 2H, 1'-CH_2, J = 7.4 Hz), 6.21 (s, 1H, OH), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 6.84 (d, 1H, C_8-H, J = 2.3 Hz) and 8.59 (d, 1H, C_5-H, J = 8.9 Hz); Found C, 77.11; H, 10.20; $C_{24}H_{36}O_3$ requires C, 77.38; H, 9.74%.

8.5.30 4-Methyl 3-tetradecylcoumarin-7-*O*-sulphamate (86)

Compound **85** (300 mg; 0.81 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to

2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **86** as white fine crystals (3 mg; 0.8 %). R_f: 0.62 ($CHCl_3$/ethyl acetate, 4:1); mp 119-121°C; MS (FAB^+) *m/z*: 452.3 [100, $(M+H)^+$], 373.3 [10, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 450.2 [100, $(M-H)^-$], 371.3 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 452.2455, $C_{24}H_{38}NO_5S$ requires 452.2471; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.22-1.47 (m, 24H, 12×CH_2), 2.41 (s, 3H, C_4-CH_3), 2.57 (t, 2H, 1'-CH_2, J = 7.8 Hz), 7.22 (d, 1H, C_8-H, J = 2.3 Hz), 7.26 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.79 (d, 1H, C_5-H, J = 8.9 Hz) and 8.20 (s, 2H, NH_2); Found C, 63.74; H, 7.99; N, 3.36 $C_{24}H_{37}NO_5S$ requires C, 63.83; H, 8.26; N, 3.10%.

8.5.31 Ethyl 2-acetylheptadecanoate (87)

Prepared by method 8.2.2, using K_2CO_3 (5.7 g; 41 mmol), water (60 ml), 1-bromopentadecane (5.0 ml; 17 mmol), ethyl 3-oxobutanoate (2.2 ml; 17 mmol), CH_2Cl_2 (60 ml) and Bu_4NCl (~10 g; 34 mmol). The crude oily residue was purified by distillation under reduced pressure to give **87** as pale yellow oil (1.78 g; 31%). R_f: 0.67 ($CHCl_3$); $bp_{0.4}$ 198-202°C; MS (FAB^+) *m/z*: 341.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 339.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 341.3058, $C_{21}H_{40}O_3$ requires 341.3056; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.26-1.79 (m, 29H, $CH_2C\underline{H}_3$ and 16×CH_2), 2.22 (s, 3H, CH_3CO), 3.53 (t, 2H, 3-H_2, J = 6.7 Hz), 3.37 (t, 1H, 2-H, J = 7.0 Hz) and 4.18 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz).

8.5.32 7-Hydroxy-4-methyl-3-pentadecylcoumarin (88)

Prepared by method 8.2.4, using resorcinol (486 mg; 4.41 mmol), **87** (1.5g; 4.5 mmol) and a mixture of CF_3COOH (0.7 ml; 8.8 mmol) and conc. H_2SO_4 (0.5 ml; 8.8 mmol). The crude brown sticky solid was purified by flash chromatography ($CHCl_3$/acetone gradient, 8:1 to 4:1) and the off-white waxy solid isolated was recrystallised from acetone/hexane to give **88** as a white soft solid (123 mg; 0.07%). R_f: 0.86 ($CHCl_3$/acetone, 3:1); mp 59-61°C; MS (FAB^+) *m/z*: 387.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 385.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 387.2892, $C_{25}H_{39}O_3$ requires 387.2899; ^1H

NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 3H, CH_3, J = 7.0 Hz), 1.11-1.80 (m, 26H, 13×CH_2), 2.38 (s, 3H, C_4-CH_3), 2.53 (t, 2H, 1'-CH_2, J = 6.6 Hz), 5.75 (s, 1H, OH), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 6.84 (d, 1H, C_8-H, J = 2.3 Hz) and 7.48 (d, 1H, C_5-H, J = 8.6 Hz).

8.5.33 4-Methyl-3-pentadecylcoumarin-7-*O*-sulphamate (89)

Compound **88** (90 mg; 0.23 mmol) was sulphamoylated by method A (8.2.5). The a crude white solid was purified by preparative TLC ($CHCl_3$/ethyl acetate gradient, 6:1) and the white solid isolated was recrstallised from ethyl acetate/hexane to give **89** as white fine solid (21 mg; 19%). R_f: 0.64 ($CHCl_3$/ethyl acetate, 4:1); mp 114-116°C; MS (FAB^+) *m/z*: 466.3 [100, $(M+H)^+$], 387.3 [10, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 464.2 [100, $(M-H)^-$], 385.3 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 466.2617, $C_{25}H_{40}NO_5S$ requires 466.2626; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 3H, CH_3, J = 7.0 Hz), 1.14-1.44 (m, 26H, 13×CH_2), 2.43 (s, 3H, C_4-CH_3), 2.58 (t, 2H, 1'-CH_2, J = 7.4 Hz), 7.25 (d, 1H, C_8-H, J = 2.3 Hz), 7.27 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.87 (d, 1H, C_5-H, J = 8.6 Hz) and 8.16 (s, 2H, NH_2); Found C, 64.00; H, 8.82; N, 3.26; $C_{25}H_{39}NO_5S$ requires C, 64.48; H, 8.44; N, 3.01%.

8.6 Other functionalities at the C-3 position of the coumarin ring

8.6.1 3-Chloro-7-hydroxy-4-methylcoumarin (90)

Prepared by method 8.2.4, using resorcinol (2.0 g; 18 mmol), ethyl 2-chloro-3-oxobutanoate (2.99 g; 18 mmol) and a mixture of CF_3COOH (2.27 ml; 36.3 mmol) and conc. H_2SO_4 (1.83 ml; 36.3 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the yellow solid isolated was recrystallised from acetone/hexane to give **90** as off-white crystals (692 mg; 18%). R_f: 0.72 ($CHCl_3$/acetone, 3:1); mp 250–253°C ([426]Lit. mp 250°C); MS (FAB^+) *m/z*: 211.1 [100, $(M(^{35}Cl)+H)^+$]; MS (FAB^-) *m/z*: 209.1 [100, $(M(^{35}Cl)-H)^-$]; Acc. MS (FAB^+) *m/z*: 211.0178 $C_{10}H_8{}^{35}ClO_3$ requires 211.0162 and 213.0152 $C_{10}H_8{}^{37}ClO_3$ requires 213.0132; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.09 (s, 3H, CH_3), 6.76 (d, 1H, C_8-H, J

= 2.4 Hz), 6.86 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.69 (d, 1H, C_5-H, J = 8.8 Hz) and 10.68 (s, 1H, OH); Found C, 57.30; H, 3.39; $C_{10}H_7ClO_3$ requires C, 57.03; H, 3.35%.

8.6.2 3-Chloro-4-methylcoumarin-7-*O*-sulphamate (91)

Compound **90** (400 mg; 1.9 mmol) was sulphamoylated by method A (8.2.5). The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **91** as white fine crystals (164 mg; 30%). R_f: 0.30 ($CHCl_3$/ethyl acetate, 4:1); mp 182–186°C; MS (FAB^+) *m/z*: 290.0 [100, $(M(^{37}Cl)+H)^+$]; MS (FAB^-) *m/z*: 288.1 [100, $(M(^{35}Cl)-H)^-$], 209.1 [50, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+) *m/z*: 290.9860, $C_{10}H_9{}^{37}ClNO_5S$ requires 290.9782 and 288.9814 $C_{10}H_9{}^{35}ClNO_5S$ requires 291.9813; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.59 (s, 3H, CH_3), 7.35 (dd, 1H, C_6-H, J = 2.1, 8.8 Hz), 7.39 (d, 1H, C_8-H, J = 2.1 Hz), 7.98 (d, 1H, C_5-H, J = 8.8 Hz) and 8.29 (s, 2H, NH_2); Found C, 41.50; H, 2.62; N, 4.64; $C_{10}H_8ClNO_5S$ requires C, 41.46; H, 2.78; N, 4.84%.

8.6.3 4-Methyl-3-phenyl-7-hydroxycoumarin (92)

Prepared by method 8.2.4, using resorcinol (2.13 g; 19.4 mmol), ethyl 3-oxo-2-phenylbutanoate (4.0 g; 19 mmol) and a mixture of CF_3COOH (3.0 ml; 39 mmol) and conc. H_2SO_4 (2.0 ml; 39 mmol). The crude yellow solid was purified by recrystallisation from ethanol to give **92** as yellow needles (4.1 g; 83%). R_f: 0.59 ($CHCl_3$/acetone, 3:1); mp 226–228°C; (^{427}Lit. mp 226-228°C); MS (FAB^+) *m/z*: 505.1 [10, $(2M+H)^+$], 253.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 503.1 [10, $(2M-H)^-$], 251.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 253.0798, $C_{16}H_{12}O_3$ requires 253.0786; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.21 (s, 3H, CH_3), 6.75 (d, 1H, C_8-H, J = 2.4 Hz), 6.84 (dd, 1H, C_6-H, J = 2.4, 8.6 Hz), 7.27-7.47 (m, 5H, Ph-H), 7.66 (d, 1H, C_5-H, J = 8.8 Hz) and 10.67 (s, 1H, OH); Found C, 76.10; H, 4.84; $C_{16}H_{12}O_3$ requires C, 76.18; H, 4.79%.

8.6.4 4-Methyl-3-phenylcoumarin-7-*O*-sulphamate (93)

Compound **92** (400 mg; 1.6 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **93** as white fine crystals (246 mg; 52%). R_f: 0.36 ($CHCl_3$/ethyl acetate, 4:1); mp 184–187°C; MS (FAB^+) *m/z*: 332.0 [100, $(M+H)^+$], 253.0 [15, $(M+H\text{-}HNSO_2)^+$]; MS (FAB^-) *m/z*: 330.1 [100, $(M-H)^-$], 251.1 [60, $(M\text{-}H_2NSO_2)^-$]; Acc. MS (FAB^+): 332.0589, $C_{16}H_{14}NO_5S$ requires 332.0593; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.28 (s, 3H, CH_3), 7.31-7.38 (m, 2H, C_6-H and C_8-H), 7.40-7.49 (m, 5H, Ph-H), 7.95 (d, 1H, C_5-H, J = 8.8 Hz) and 8.27 (s, 2H, NH_2); Found C, 58.00; H, 3.94; N, 4.21; $C_{16}H_{13}NO_5S$ requires C, 58.00; H, 3.95; N, 4.23%.

8.6.5 3-Benzyl-4-methyl-7-hydroxycoumarin (94)

Prepared by method 8.2.4, using resorcinol (1.99 g; 18.2 mmol), ethyl 2-benzyl-3-oxobutanoate (4.0 g; 18 mmol) and a mixture of CF_3COOH (2.8 ml; 36 mmol) and conc. H_2SO_4 (1.8 ml; 36 mmol). The crude brown solid was purified by recrystallisation from ethanol to give **94** as white crystals (4.35 g; 90%). R_f: 0.79 ($CHCl_3$/acetone, 3:1); mp 230–232°C ([428]Lit. mp 226-227°C); MS (FAB^+) *m/z*: 532.9 [10, $(2M+H)^+$], 267.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 265.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 267.1026, $C_{17}H_{15}O_3$ requires 267.1012; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.39 (s, 3H, CH_3), 3.92 (s, 2H, CH_2Ph), 6.71 (d, 1H, C_8-H, J = 2.4 Hz), 6.80 (dd, 1H, C_6-H, J = 2.4, 8.8 Hz), 7.15-7.28 (m, 5H, Ph-H), 7.64 (d, 1H, C_5-H, J = 8.8 Hz) and 10.48 (s, 1H, OH); Found C, 76.60; H, 5.34; $C_{17}H_{14}O_3$ requires C, 76.68; H, 5.30%.

8.6.6 3-Benzyl-4-methylcoumarin-7-*O*-sulphamate (95)

Compound **94** (400 mg; 1.5 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **95** as white fine crystals (279 mg; 54%). R_f: 0.57 ($CHCl_3$/ethyl acetate, 4:1); mp 168–170°C; MS (FAB^+) *m/z*: 346.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 344.1 [100, $(M-H)^-$], 265.1 [60,

$(M\text{-}H_2NSO_2)^-$]; Acc. MS (FAB^+): 346.0755, $C_{17}H_{16}NO_5S$ requires 346.0749; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.39 (s, 3H, CH_3), 3.99 (s, 2H, $PhCH_2$), 7.17-7.31 (m, 6H, C_6-H and Ph-H), 7.32 (d, 1H, C_8-H, J = 2.0 Hz), 7.92 (d, 1H, C_5-H, J = 8.6 Hz) and 8.22 (s, 2H, NH_2); Found C, 59.10; H, 4.39; N, 4.04; $C_{17}H_{15}NO_5S$ requires C, 59.12; H, 4.38; N, 4.06%.

8.6.7 Ethyl 2-acetyl-4-phenylbutanoate (96)

Prepared by method 8.2.2, using K_2CO_3 (8.97 g; 64.85 mmol), water (50 ml), (2-bromoethyl)benzene (5.0 ml; 27 mmol), ethyl 3-oxobutanoate (3.44 ml; 27 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 27 mmol). The crude oily residue was purified by flash chromatography ($CHCl_3$) to give **96** as a pale yellow oil (2.17 g; 34%). ([429]Lit. $bp_{1.4}$ 167-168°C); R_f: 0.78 ($CHCl_3$); MS (FAB^+) *m/z*: 235.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 235.1335, $C_{14}H_{19}O_3$ requires 235.1334; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.25 (t, 3H, $CH_2C\underline{H}_3$, J = 6.7 Hz), 2.29 (s, 3H, CH_3), 3.02 (q, 2H, $C\underline{H}_2CH_2Ph$, J = 7.1 Hz), 3.71 (t, 1H, CH_2, J = 7.3 Hz), 3.95 (t, 2H, CH_2Ph, J = 7.1 Hz), 4.11 (q, 2H, $C\underline{H}_2CH_3$, J = 7.1 Hz) and 7.16-7.34 (m, 5H, Ph-H).

8.6.8 7-Hydroxy-4-methyl-3-(2-phenylethyl)coumarin (97)

Prepared by method 8.2.4, using resorcinol (705 mg; 6.4 mmol), **96** (1.5g; 6.4 mmol) and a mixture of CF_3COOH (1 ml; 13 mmol) and conc. H_2SO_4 (0.7 ml; 13 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone gradient, 8:1 to 4:1) and the white solid isolated was recrystallised from acetone/hexane to give **97** as grey crystals (746 mg; 42%). R_f: 0.80 ($CHCl_3$/acetone, 3:1); mp 175–178°C; MS (FAB^+) *m/z*: 561.1 [10, $(2M+H)^+$], 281.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 279.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 281.1182, $C_{18}H_{17}O_3$ requires 281.1178; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.16 (s, 3H, CH_3), 2.73 (t, 2H, $C\underline{H}_2CH_2Ph$, J = 5.5 Hz), 2.79 (t, 2H, $PhCH_2$, J = 5.2 Hz), 6.69 (d, 1H, C_8-H, J = 2.4 Hz), 6.78 (dd, 1H, C_6-H, J = 2.4, 8.5 Hz), 7.16-7.29 (m, 5H, Ph-H), 7.56 (d, 1H, C_5-H, J = 8.8 Hz) and 10.42 (s, 1H, OH); Found C, 77.35; H, 5.95; $C_{18}H_{16}O_3$ requires C, 77.12; H, 5.75%.

8.6.9 4-Methyl-3-(2-phenylethyl)coumarin-7-*O*-sulphamate (98)

Compound **97** (400 mg; 1.43 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **98** as white fine crystals (132 mg; 26%). R_f: 0.54 ($CHCl_3$/ethyl acetate, 4:1); mp 196–198°C; MS (FAB^+) *m/z*: 360.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 358.0 [100, $(M-H)^-$], 279.1 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 360.0917, $C_{18}H_{18}NO_5S$ requires 360.0906; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.21 (s, 3H, CH_3), 2.76 (t, 2H, $C\underline{H}_2CH_2Ph$, J = 7.3 Hz), 2.87 (t, 2H, $PhCH_2$, J = 7.9 Hz), 7.17-7.27 (m, 7H, C_6-H, C_8-H and Ph-H), 7.83 (d, 1H, C_5-H, J = 8.8 Hz) and 8.19 (s, 2H, NH_2); Found C, 60.00; H, 4.81; N, 3.89; $C_{18}H_{17}NO_5S$ requires C, 60.16; H, 4.77; N, 3.90%.

8.6.10 Ethyl 2-acetyl-5-phenylpentanoate (99)

Prepared by method 8.2.2, using K_2CO_3 (16.7 g; 0.12 mol), water (60 ml), 1-bromo-3-phenylpropane (10.0 ml; 50.2 mmol), ethyl 3-oxobutanoate (6.5 ml; 50.2 mmol), CH_2Cl_2 (60 ml) and Bu_4NCl (28 g; 0.1 mol). The crude oily residue was purified by flash chromatography ($CHCl_3$/hexane, 10:1) to give **99** as a colourless oil (4.92 g; 39%). R_f: 0.61 ($CHCl_3$/hexane, 10:1); MS (FAB^+) *m/z*: 249.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 247.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 249.1491, $C_{15}H_{21}O_3$ requires 249.1491; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.26 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 2.19 (s, 3H, CH_3), 1.85-2.61 (m, 6H, CH_2), 2.64 (t, 1H, 2-H, J = 7.8 Hz), 4.21 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz) and 7.15-7.29 (m, 5H, Ph-H).

8.6.11 7-Hydroxy-4-methyl-3-[3-phenylpropyl]coumarin (100)

Prepared by method 8.2.4, using resorcinol (443 mg; 4.03 mmol), **99** (1.0 g; 4.0 mmol) and a mixture of CF_3COOH (0.6 ml; 8.1 mmol) and conc. H_2SO_4 (0.4 ml; 8.1 mmol). The crude orange solid was purified by flash chromatography ($CHCl_3$/acetone gradient, 8:1 to 4:1) and the white solid isolated was recrystallised from THF/hexane to give **100** as pale green crystals (275 mg; 22%). R_f: 0.76 ($CHCl_3$/acetone, 3:1); mp 189-191°C; MS (FAB^+) *m/z*: 589.2 [10, $(2M+H)^+$], 295.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 293.1

[100, $(M-H)^-$]; Acc. MS (FAB^+): 295.1675, $C_{19}H_{19}O_3$ requires 295.1680; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.71 (quintet, 2H, $CH_2C\underline{H}_2CH_2Ph$, J = 7.8 Hz), 2.13 (s, 3H, CH_3), 2.54 (t, 2H, $C\underline{H}_2CH_2CH_2Ph$, J = 7.8 Hz), 2.63 (t, 2H, $CH_2CH_2C\underline{H}_2Ph$, J = 7.4 Hz), 6.82 (d, 1H, C_8-H, J = 2.4 Hz), 7.13-7.27 (m, 6H, C_6-H and Ph-H), 7.42 (d, 1H, C_5-H, J = 8.8 Hz) and 10.25 (s, 1H, OH); Found C, 77.13; H, 6.28; $C_{19}H_{18}O_3$ requires C, 77.53; H, 6.16%.

8.6.12 4-Methyl-3-[3-phenylpropyl]coumarin-7-*O*-sulphamate (101)

Compound **100** (230 mg; 0.78 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate gradient, 8:1 to 2:1) and the white solid isolated was recrystallised from THF/hexane to give **101** as white crystals (62 mg; 21%). R_f: 0.54 ($CHCl_3$/ethyl acetate, 4:1); mp 154–156°C; MS (FAB^+) *m/z*: 374.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 372.1 [100, $(M-H)^-$], 293.1 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 374.1060, $C_{19}H_{20}NO_5S$ requires 374.1062; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.76 (pentet, 2H, $CH_2C\underline{H}_2CH_2Ph$, J = 8.2 Hz), 2.49 (s, 3H, CH_3), 2.62 (t, 2H, $C\underline{H}_2CH_2CH_2Ph$, J = 8.2 Hz), 2.67 (t, 2H, $CH_2CH_2C\underline{H}_2Ph$, J = 7.4 Hz), 7.15-7.39 (m, 7H, C_8-H, C_6-H and Ph-H), 7.87 (d, 1H, C_5-H, J = 8.8 Hz) and 8.19 (s, 2H, NH_2); Found C, 61.30; H, 4.94; N, 3.49; $C_{19}H_{19}NO_5S$ requires C, 61.11; H, 5.13; N, 3.75%.

8.6.13 Ethyl 2-(cyclohexylmethyl)-3-oxobutanoate (102)

Prepared by method 8.2.2, using K_2CO_3 (7.5 g; 54 mmol), water (50 ml), cyclohexylmethyl bromide (4.0 ml; 23 mmol), ethyl 3-oxobutanoate (2.9 ml; 23 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (~10 g; 23 mol). The crude pale yellow oil was purified by flash chromatography ($CHCl_3$) to give **102** as a pale yellow oil (1.44 g; 28%). ([430]Lit. bp_{19} 166°C); R_f: 0.58 ($CHCl_3$); MS (FAB^+) *m/z*: 227.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 225.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 227.1632, $C_{13}H_{23}O_3$ requires 227.1647; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.08-1.34 (m, 11H, cyclohexyl H), 1.27 (t,

3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 1.69 (t, 2H, CH_2, J = 7.0 Hz), 2.22 (s, 3H, CH_3), 3.53 (t, 1H, 2-H, J = 7.4 Hz) and 4.21 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz).

8.6.14 3-Cyclohexylmethyl-7-hydroxy-4-methylcoumarin (103)

Prepared by method 8.2.4, using resorcinol (487 mg; 4.42 mmol), **102** (1.0 g; 4.4 mmol) and a mixture of CF_3COOH (0.7 ml; 8.8 mmol) and conc. H_2SO_4 (0.5 ml; 8.8 mmol). The crude orange solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **103** as fine white crystals (409 mg; 34%). R_f: 0.74 ($CHCl_3$/acetone, 3:1); mp 193-196°C; MS (FAB^+) *m/z*: 545.3 [15, $(2M+H)^+$], 273.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 271.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 273.1491, $C_{17}H_{21}O_3$ requires 273.1492; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.11-1.91 (m, 11H, cyclohexyl H), 2.35 (s, 3H, CH_3), 2.43 (d, 2H, CH_2, J = 7.0 Hz), 6.68 (d, 1H, C_8-H, J = 2.3 Hz), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.59 (d, 1H, C_5-H, J = 8.6 Hz) and 10.21 (s, 1H, OH); Found C, 74.80; H, 7.47; $C_{17}H_{20}O_3$ requires C, 74.97; H, 7.40%.

8.6.15 3-Cyclohexylmethyl-4-methylcoumarin-7-*O*-sulphamate (104)

Compound **103** (300 mg; 1.10 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **104** as white crystals (46 mg; 12%). R_f: 0.55 ($CHCl_3$/ethyl acetate, 4:1); mp 170–171°C; MS (FAB^+) *m/z*: 352.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 350.0 [100, $(M-H)^-$], 271.1 [45, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 352.1214, $C_{17}H_{22}NO_5S$ requires 352.1218; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.22-1.31 (m, 11H, cyclohexyl H), 2.06 (s, 3H, CH_3), 2.58 (d, 2H, CH_2, J = 7.0 Hz), 7.32-7.35 (m, 2H, C_8-H and C_6-H), 7.93 (d, 1H, C_5-H, J = 8.6 Hz) and 8.39 (s, 2H, NH_2); Found C, 58.30; H, 5.86; N, 3.79; $C_{17}H_{21}NO_5S$ requires C, 58.10; H, 6.02; N, 3.99%.

8.6.16 Ethyl 2-acetyl-4-cyclohexylbutanoate (105)

Prepared by method 8.2.2, using K_2CO_3 (8.68 g; 62.8 mmol), water (60 ml), 1-bromo-2-cyclohexylethane (5.0 ml; 26.2 mmol), ethyl 3-oxobutanoate (3.34 ml; 26.2 mmol), CH_2Cl_2 (50 ml) and Bu_4NCl (7.3 g; 26 mmol). The crude orange oily residue was purified by flash chromatography ($CHCl_3$) to give **105** as a pale yellow oil (850 mg; 14%). ([430]Lit. bp_{19} 175°C); R_f: 0.72 ($CHCl_3$); MS (FAB^+) *m/z*: 241.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 239.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 241.1804, $C_{14}H_{25}O_3$ requires 241.1804; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.19-1.66 (m, 13H, cyclohexyl H and CH_2), 1.27 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 1.80-1.87 (m, 2H, CH_2), 2.22 (s, 3H, CH_3), 3.35 (t, 1H, 2-H, J = 7.4 Hz) and 4.19 (q, 2H, $C\underline{H}_2CH_3$, J = 7.0 Hz).

8.6.17 3-(2-Cyclohexylethyl)-4-methyl-7-hydroxycoumarin (106)

Prepared by method 8.2.4, using resorcinol (343 mg; 3.12 mmol), **105** (750 mg; 3.12 mmol) and a mixture of CF_3COOH (0.5 ml; 6.2 mmol) and conc. H_2SO_4 (0.4 ml; 6.2 mmol). The crude orange solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **106** as white crystals (352 mg; 39%). R_f: 0.79 ($CHCl_3$/acetone, 3:1); mp 148-151°C; MS (FAB^+) *m/z*: 572.9 [10, $(2M+H)^+$], 287.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 571.1 [10, $(2M-H)^-$], 285.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 287.1639, $C_{18}H_{23}O_3$ requires 287.1647; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.11-1.73 (m, 11H, cyclohexyl H), 2.34 (s, 3H, CH_3), 2.51-2.53 (m, 4H, CH_2CH_2), 6.67 (d, 1H, C_8-H, J = 2.3 Hz), 6.78 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.59 (d, 1H, C_5-H, J = 8.9 Hz) and 10.38 (s, 1H, OH); Found C, 75.50; H, 7.67; $C_{18}H_{22}O_3$ requires C, 75.50; H, 7.74%.

8.6.18 3-(2-Cyclohexylethyl)-4-methylcoumarin-7-*O*-sulphamate (107)

Compound **106** (250 mg; 0.87 mmol) was sulphamoylated by method 8.2.5 A. The crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **107** as white fine crystals (155 mg; 49%). R_f: 0.47 ($CHCl_3$/ethyl acetate, 4:1); mp 179-181°C; MS (FAB^+) *m/z*: 366.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 364.0 [100,

$(M-H)^-$], 285.1 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 366.1319, $C_{18}H_{24}NO_5S$ requires 366.1297; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.19-1.78 (m, 11H, cyclohexyl H), 2.41 (s, 3H, CH_3), 2.49-2.59 (m, 4H, CH_2CH_2), 7.25-7.28 (m, 2H, C_8-H and C_6-H), 7.87 (d, 1H, C_5-H, J = 8.6 Hz) and 8.20 (s, 2H, NH_2); Found C, 59.10; H, 6.31; N, 3.62; $C_{18}H_{23}NO_5S$ requires C, 59.16; H, 6.34; N, 3.83%.

8.7 Other substituted coumarins

8.7.1 Ethyl 2-nonyl-3-oxododecanoate (108)

Prepared by method 8.2.2, using K_2CO_3 (6.85 g; 49.5 mmol), water (60 ml), **16** (5.0 g; 21 mmol), nonyl bromide (4.3 ml; 21 mmol), CH_2Cl_2 (60 ml) and Bu_4NCl (~6.0 g; 21 mmol). The crude yellow oily residue was purified by flash chromatography ($CHCl_3$) to give **108** as a pale yellow oil. (2.6 g; 34%). R_f: 0.89 ($CHCl_3$); MS (FAB^+) *m/z*: 369.4 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 367.4 [100, $(M-H)^-$]; Acc. MS (FAB^+): 369.3370, $C_{23}H_{45}O_3$ requires 369.3369; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (t, 6H, two terminal CH_3, J = 7.4 Hz), 1.24-1.89 (m, 33H, $CH_2C\underline{H}_3$ and 15×CH_2), 2.73 (t, 2H, 4-CH_2, J = 7.4 Hz), 3.41 (t, 1H, 2-H, J = 7.4 Hz) and 4.18 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz).

8.7.2 3,4-Dinonyl-7-hydroxycoumarin (109)

Prepared by method 8.2.4, using resorcinol (597 mg; 5.4 mmol), **108** (4.0 g; 5.4 mmol) and a mixture of CF_3COOH (0.8 ml; 11 mmol) and conc. H_2SO_4 (0.5 ml; 11 mmol). The crude brown residue was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) to give **109** as a thick yellow syrup, which solidified on standing (874 mg; 39%). This waxy solid could not be recrystallised. R_f: 0.90 ($CHCl_3$/acetone, 3:1); mp 49-51°C; MS (FAB^+) *m/z*: 415.4 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 413.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 415.3206, $C_{27}H_{43}O_3$ requires 415.3212; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.85 (t, 6H, two terminal CH_3, J = 7.4 Hz), 1.24-1.62 (m, 28H, 14×CH_2), 2.46 (t, 2H, C_3-CH_2, J = 7.4 Hz), 2.72-2.74 (m, 2H, C_4-CH_2), 6.67 (d, 1H, C_8-H, J = 2.3 Hz), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.58 (d, 1H, C_5-H, J = 8.9 Hz) and 10.41 (s, 1H, OH).

8.7.3 3,4-Dinonylcoumarin-7-*O*-sulphamate (110)

Upon sulphamation (method A 8.2.5), compound **109** (300 mg; 0.72 mmol) gave a crude white solid, which was fractionated by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **110** as white fine crystals (101 mg; 28%). R_f: 0.71 ($CHCl_3$/ethyl acetate, 6:1); mp 86-87°C; MS (FAB^+) *m/z*: 494.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 492.2 [100, $(M-H)^-$], 413.3 [15, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 494.2652, $C_{27}H_{44}NO_5S$ requires 494.2677; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88 (t, 6H, two terminal CH_3, J = 7.2 Hz), 1.22-1.58 (m, 28H, 14×CH_2), 2.44-2.47 (m, 2H, C_3-CH_2), 2.70-2.78 (m, 2H, C_4-CH_2), 6.61 (d, 1H, C_8-H, J = 2.3 Hz), 6.78 (dd, 1H, C_6-H, J = 2.3, 8.8 Hz), 7.57 (d, 1H, C_5-H, J = 8.9 Hz) and 8.23 (s, 2H, NH_2).

8.7.4 6-Ethyl-7-hydroxy-4-methyl-3-[3-phenylpropyl]coumarin (111)

Prepared by method 8.2.4, using 4-ethylresorcinol (556 mg; 4.03 mmol), **99** (1.0 g; 4.0 mmol) and a mixture of CF_3COOH (0.6 ml; 8.1 mmol) and conc. H_2SO_4 (0.4 ml; 8.1 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from acetone/hexane to give **111** as white crystals (897 mg; 69%). R_f: 0.80 ($CHCl_3$/acetone, 3:1); mp 146–149°C; MS (FAB^+) *m/z*: 323.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 321.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 323.1644, $C_{21}H_{23}O_3$ requires 323.1647; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.25 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.85 (quintet, 2H, $CH_2C\underline{H}_2CH_2Ph$, J = 7.8 Hz), 2.31 (s, 3H, CH_3), 2.66-2.74 (m, 6H, $C\underline{H}_2CH_3$ and $C\underline{H}_2CH_2C\underline{H}_2Ph$), 6.72 (s, 1H, OH) and 7.06-7.32 (m, 7H, C_8-H, Ph-H and C_5-H); Found C, 77.90; H, 6.55; $C_{21}H_{22}O_3$ requires C, 78.23; H, 6.88%.

8.7.5 6-Ethyl-4-methyl-3-[3-phenylpropyl]coumarin-7-*O*-sulphamate (112)

Upon sulphamoylation (Method 8.2.5 A), compound **111** (250 mg; 0.85 mmol) gave a crude white solid, which was fractionated by flash chromatography ($CHCl_3$/ethyl acetate gradient, 8:1 to 2:1). The white solid that isolated was recrystallised from THF/hexane to give **112** as white fine crystals (184 mg; 54%). R_f: 0.54 ($CHCl_3$/ethyl

acetate, 4:1); mp 199–201°C; MS (FAB^+) *m/z*: 402.3 [100, $(M+H)^+$], 295.1 [15, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 400.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 402.1376, $C_{21}H_{24}NO_5S$ requires 402.1375; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.17 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.72 (quintet, 2H, $CH_2C\underline{H}_2CH_2Ph$, J = 7.8 Hz), 2.36 (s, 3H, CH_3), 2.56-2.69 (m, 6H, $C\underline{H}_2CH_3$ and $C\underline{H}_2CH_2C\underline{H}_2Ph$), 7.13-7.31 (m, 7H, C_8-H, Ph-H and C_5-H) and 7.66 (s, 2H, NH_2).

8.7.6 4,8-Dimethyl-7-hydroxy-3-[3-phenylpropyl]coumarin (113)

Prepared by method 8.2.4, using 2-methylresorcinol (499 mg; 4.03 mmol), **99** (1 g; 4 mmol) and a mixture of CF_3COOH (0.7 ml; 8.1 mmol) and conc. H_2SO_4 (0.5 ml; 8.1 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from THF/hexane to give **113** as fine pale yellow crystals (1.03 g; 83%). R_f: 0.85 ($CHCl_3$/acetone, 4:1); mp 191-192°C; MS (FAB^+) *m/z*: 617.3 [10, $(2M+H)^+$], 309.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 615.2 [10, $(2M-H)^-$], 302.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 309.1492, $C_{20}H_{21}O_3$ requires 309.1491; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.74 (quintet, 2H, $CH_2C\underline{H}_2CH_2Ph$, J = 8.2 Hz), 2.15 (s, 3H, C_4-CH_3), 2.29 (s, 3H, C_8-CH_3), 2.58 (t, 2H, $C\underline{H}_2CH_2CH_2Ph$, J = 7.4 Hz), 2.65 (t, 2H, CH_2Ph, J = 7.4 Hz), 6.84 (d, 1H, C_6-H, J = 8.6 Hz), 7.15-7.29 (m, 5H, Ph-H), 7.43 (d, 1H, C_5-H, J = 8.6 Hz) and 10.28 (s, 1H, OH); Found C, 77.40; H, 6.62; $C_{20}H_{20}O_3$ requires C, 77.90; H, 6.54%.

8.7.7 4,8-Dimethyl-3-[3-phenylpropyl]coumarin-7-*O*-sulphamate (114)

Upon sulphamoylation (method 8.2.5 A), compound **113** (300 mg; 0.97 mmol) gave a crude white solid, which was fractionated by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **114** as white fine crystals (145 mg; 39%). R_f: 0.44 ($CHCl_3$/ethyl acetate, 4:1); mp 153-154°C; MS (FAB^+) *m/z*: 388.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 386.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 388.1146, $C_{20}H_{22}NO_5S$ requires 388.1140; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.75 (quintet, 2H, $CH_2C\underline{H}_2CH_2Ph$, J = 7.0 Hz), 2.32

(s, 3H, C_4-CH_3), 2.37 (s, 3H, C_8-CH_3), 2.60-2.69 (m, 4H, C$\underline{H_2}$CH_2C$\underline{H_2}$Ph), 7.15-7.29 (m, 5H, Ph-H), 7.31 (d, 1H, C_6-H, J= 8.6 Hz), 7.71 (d, 1H, C_5-H, J= 8.6 Hz) and 8.22 (s, 2H, NH_2); Found C, 61.50; H, 5.41; N, 3.58; $C_{20}H_{21}NO_5S$ requires C, 62.00; H, 5.46; N, 3.62%.

8.7.9 7-Hydroxy-3-(4-methoxyphenyl)-4-methylcoumarin (115)

To a CH_2Cl_2 (100 ml) solution of 2,4-dihydroxyacetophenone (5.0 g; 33 mmol; 1 eq.), tetrabutylammonium hydrogensulphate (300 mg; 0.88 mmol; 0.03 eq.) and 20% aq. K_2CO_3 (100 ml) was added 4-methoxyphenylacetyl chloride (6.7 g; 36 mmol; 1.1 eq.) in CH_2Cl_2 (30 ml) dropwise during 30 min and stirred for 4 hours at R.T. The organic layer was separated, washed with water (3×200 ml), dried and concentrated. The crude pale brown syrup was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from THF/hexane to give **115** as white crystals (1.2 g; 13%). R_f: 0.68 (UV visible and fluorescent) (ethyl acetate/hexane, 1:1); mp 230-232°C ([431]Lit. mp 233-234°C); MS (FAB^+) *m/z*: 283.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 281.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 283.0898, $C_{17}H_{15}O_4$ requires 283.0892; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 2.60 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 6.63 (dd, 1H, C_6-H, J= 2.3, 8.9 Hz), 6.71 (d, 1H, C_8-H, J= 2.3 Hz), 6.90 (d, 2H, Ph-2,6-H_2, J= 8.6 Hz), 7.29 (d, 2H, Ph-3,5-H_2, J= 8.6 Hz), 7.72 (d, 1H, C_5-H, J = 8.9 Hz) and 12.42 (s, 1H, OH); Found C, 71.98; H, 5.36; $C_{17}H_{14}O_4$ requires C, 72.33; H, 5.00%.

8.7.10 3-(4-Methoxyphenyl)-4-methylcoumarin-7-*O*-sulphamate (116)

Upon sulphamoylation by method 8.2.5 B, compound **115** (500 mg; 1.77 mmol) gave a crude white solid, which was fractionated by flash chromatography ($CHCl_3$/ethyl acetate 8:1 to 2:1 gradient,). The white solid isolated was recrystallised from ethyl acetate/hexane to give **116** as white fine leaves (331 mg; 52%). R_f: 0.86 ($CHCl_3$/ethyl acetate, 4:1); mp 129-132°C; MS (FAB^+) *m/z*: 362.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 361.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 362.0705, $C_{17}H_{16}NO_6S$ requires 362.0698; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 2.77 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 6.93 (d, 2H,

Ph-2,6-H_2, J= 8.6 Hz), 7.31 (d, 2H, Ph-3,5-H_2, J= 8.6 Hz), 7.35 (dd, 1H, C_6-H, J= 1.9, 8.9 Hz), 7.48 (d, 1H, C_8-H, J= 1.9 Hz), 8.21 (d, 1H, C_5-H, J = 8.9 Hz), 8.24 (s, 2H, NH_2); Found C, 56.30; H, 4.21; N, 3.78; $C_{17}H_{15}NO_6S$ requires C, 56.50; H, 4.18; N, 3.88%.

8.8 Tricyclic coumarin sulphamates

8.8.1 Ethyl 2-oxocyclononanecarboxylate (117)

Prepared by method 8.2.3, by stirring a mixture of NaH (1.71 g, 42.8 mmol), diethyl carbonate (80 ml) and cyclononanone (3.0 g, 21 mmol). The crude yellow oily residue was purified by distillation under reduced pressure to give **117** as clear oil (4.15 g, 91%). R_f: 0.72 ($CHCl_3$); $bp_{0.3}$ 146-150°C; ([432]Lit. bp_2 108–110°C; MS (FAB^+) *m/z*: 213.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 213.1499, $C_{12}H_{21}O_3$ requires 213.1491; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.23 (t, 1.8H, keto $CH_2C\underline{H}_3$, J = 7.2 Hz), 1.30 (t, 1.2H, enol $CH_2C\underline{H}_3$, J = 7.2 Hz), 1.37-2.66 (m, 14H), 3.62 (m, 0.6H, keto CHC=O), 4.14 (q, 1.2H, keto $C\underline{H}_2CH_3$, J = 7.2 Hz), 4.21 (q, 0.8H, enol $C\underline{H}_2CH_3$, J = 7.2 Hz) and 12.76 (s, 0.4H, enol OH exchanged with D_2O).

8.8.2 3-Hydroxy-6-oxo-8,9,10,11,12,13-hexahydro-*7H*-cyclonona-[c][1]-benzopyran (118)

Prepared by method 8.2.4, using resorcinol (1.56 g; 14.1 mmol), **117** (3.0 g; 14 mmol) and a mixture of CF_3COOH (2.2 ml; 28 mmol) and conc. H_2SO_4 (1.5 ml; 28 mmol). The crude orange solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **118** as white fine crystals (909 mg; 25%). R_f: 0.82 ($CHCl_3$/acetone, 3:1); mp 197-200°C; MS (FAB^+) *m/z*: 259.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 257.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 259.1323, $C_{16}H_{19}O_3$ requires 259.1334; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.29-2.51 (m, 10H, 5×CH_2), 2.66 (t, 2H, C_8-CH_2, J = 5.8 Hz), 2.93 (t, 2H, C_{14}-CH_2, J = 6.1 Hz), 6.69 (d, 1H, C_4-H, J = 2.4 Hz), 6.78 (dd, 1H, C_2-H, J = 2.4, 8.8 Hz), 7.59 (d, 1H, C_1-H, J = 8.8 Hz) and 10.39 (s, 1H, OH); Found C, 74.10; H, 6.91; $C_{16}H_{18}O_3$

requires C, 74.40; H, 7.02%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 323.2 nm; t_R = 6.5 min.

8.8.3 6-Oxo-8,9,10,11,12,13-hexahydro-*7H*-cyclonona-[c][1]-benzopyran-3-*O*-sulphamate (119)

Compound **118** (400 mg; 1.55 mmol) was sulphamoylated by method 8.2.5 A, and the crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **119** as white fine crystals (201 mg; 38%). R_f: 0.46 ($CHCl_3$/ethyl acetate, 4:1); mp 167–168°C; MS (FAB^+) *m/z*: 338.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 336.1 [100, $(M-H)^-$], 257.1 [30, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 338.1061, $C_{16}H_{20}NO_5S$ requires 338.1062; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.84-1.74 (m, 10H, 5×CH_2), 1.52 (t, 2H, C_8-CH_2, *J* = 5.8 Hz), 1.57 (t, 2H, C_{14}-CH_2, *J* = 6.1 Hz), 7.26 (dd, 1H, C_2-H, *J* = 2.4, 8.8 Hz), 7.31 (d, 1H, C_4-H, *J* = 2.1 Hz), 7.89 (d, 1H, C_1-H, *J* = 8.8 Hz) and 8.20 (s, 2H, NH_2); Found C, 56.85; H, 5.58; N, 4.00 $C_{16}H_{19}NO_5S$ requires C, 56.96; H, 5.68; N, 4.15 %; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 284 and 312.5 nm; t_R = 3.1 min.

8.8.4 Ethyl 2-oxocyclodecanecarboxylate (120)

Prepared by method 8.2.3, by stirring a mixture of NaH (1.3 g, 32 mmol), diethyl carbonate (60 ml) and cyclodecanone (2.5g, 16 mmol). The crude pale yellow oily residue was purified by distillation under reduced pressure to give **120** as a colourless oil (2.81 g, 76%). R_f: 0.81 ($CHCl_3$); $bp_{0.23}$ 84–87°C. ([432]Lit. bp_1 118–120°C); MS (FAB^+) *m/z*: 227.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 227.1644, $C_{13}H_{23}O_3$ requires 227.1647; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.24 (t, 1.2H, keto $CH_2C\underline{H}_3$, *J* = 7.0 Hz), 1.31 (t, 1.8H, enol $CH_2C\underline{H}_3$, *J* = 7.0 Hz), 1.34-2.76 (m, 16H), 3.82-3.85 (m, 0.7H, keto CHC=O), 4.13 (q, 0.5H, keto $C\underline{H}_2CH_3$, *J* = 7.0 Hz), 4.22 (q, 1.5H, enol $C\underline{H}_2CH_3$, *J* = 7.0 Hz) and 12.98 (s, 0.3H, enol OH exchanged with D_2O).

8.8.5 3-Hydroxy-6-oxo-7,8,9,10,11,12,13,14-octahydrocycloundeca-[c][1]-benzopyran (121)

Prepared by method 8.2.4, using resorcinol (970 mg; 8.84 mmol), **120** (2.0 g; 8.8 mmol) and a mixture of CF_3COOH (1.5 ml; 18 mmol) and conc. H_2SO_4 (1.0 ml; 18 mmol). The crude dark orange solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the white solid isolated was recrystallised from THF/hexane to give **121** as white crystals (789 mg; 33%). R_f: 0.72 ($CHCl_3$/acetone, 3:1); mp 240-241°C; MS (FAB^+) *m/z*: 273.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 271.1[100, $(M-H)^-$]; Acc. MS (FAB^+): 273.1488, $C_{17}H_{21}O_3$ requires 273.1491; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.88-2.18 (m, 12H, 6×CH_2), 2.83 (t, 2H, C_7-CH_2, J = 6.7 Hz), 3.02 (t, 2H, C_{14}-CH_2, J = 6.7 Hz), 5.96 (s, 1H, OH), 6.78 (dd, 1H, C_2-H, J = 2.7, 8.5 Hz), 6.83 (d, 1H, C_4-H, J = 2.7 Hz) and 7.53 (d, 1H, C_1-H, J = 8.5 Hz); Found C, 74.30; H, 7.43; $C_{17}H_{20}O_3$ requires C, 74.94; H, 7.40%; HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 322 nm; t_R = 4.5 min.

8.8.6 6-Oxo-7,8,9,10,11,12,13,14-octahydrocyclodeca-[c][1]-benzopyran-3-*O*-sulphamate (122)

Compound **121** (400 mg; 1.47 mmol) was sulphamoylated by method 8.2.5 A, and the crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **122** as white fine crystals (235 mg; 46%). R_f: 0.71 ($CHCl_3$/ethyl acetate, 4:1); mp 183-185°C; MS (FAB^+) *m/z*: 352.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 350.1 [100, $(M-H)^-$], 271.1 [100, $(M-H_2NSO_2)$); Acc. MS (FAB^+): 352.1223, $C_{17}H_{22}NO_5S$ requires 352.1219; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.17-3.0 (m, 14H, 7×CH_2), 3.09 (t, 2H, C_{14}-CH_2, J = 6.4 Hz), 7.26 (m, 1H, C_2-CH), 7.46 (d, 1H, C_4-H, J = 1.2 Hz), 7.93 (d, 1H, C_1-H, J = 8.8 Hz) and 8.21 (s, 2H, NH_2); Found C, 58.40; H, 6.28; N, 2.63 $C_{17}H_{21}NO_5S$ requires C, 58.10; H, 6.02; N, 3.99%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 284 and 312.5 nm; t_R = 6.3 min.

8.8.7 Ethyl 2-oxocycloundecanecarboxylate (123)

Prepared by method 8.2.3, by stirring a mixture of NaH (1.19 g, 29.7 mmol), diethyl carbonate (70 ml) and cycloundecanone (2.5 g, 15 mmol). The crude yellow oily residue was purified by distillation under reduced pressure to give **123** as pale yellow oil (2.07g, 58%). R_f: 0.31 (CH_2Cl_2); $bp_{0.15}$ 103–108°C. ([432]Lit. bp_5 140–143°C; MS (FAB^+) *m/z*: 241.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 239.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 241.1806, $C_{14}H_{25}O_3$ requires 241.1804; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.20 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 1.26-2.76 (m, 19H) and 4.10 (q, 2H, $CH_3C\underline{H}_2$, J = 7.3 Hz).

8.8.8 3-Hydroxy-6-oxo-8,9,10,11,12,13,14,15-octahydro-*7H*-cycloundeca-[c][1]-benzopyran (124)

Prepared by method 8.2.4, using resorcinol (917 mg; 8.33 mmol), **123** (2.0 g; 8.3 mmol) and a mixture of CF_3COOH (2.0 ml; 17 mmol) and conc. H_2SO_4 (1.6 ml; 17 mmol). The crude yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the yellow solid isolated was recrystallised from THF/hexane to give **124** as pale yellow fine crystals (344 mg; 14%). R_f: 0.76 ($CHCl_3$/acetone, 3:1); mp 214-215°C; MS (FAB^+) *m/z*: 287.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 285.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 287.1644, $C_{18}H_{23}O_3$ requires 287.1647; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.26-1.68 (m, 14H, 7×CH_2), 2.56 (t, 2H, C_8-CH_2, J = 7.0 Hz), 2.84 (t, 2H, C_{16}-CH_2, J = 7.0 Hz), 6.67 (d, 1H, C_4-H, J = 2.1 Hz), 6.78 (dd, 1H, C_2-H, J = 2.1, 8.7 Hz), 7.63 (d, 1H, C_1-H, J = 8.8 Hz) and 10.42 (s, 1H, OH); Found C, 75.50; H, 7.75; $C_{18}H_{22}O_3$ requires C, 75.50; H, 7.74%; HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 323.2 nm; t_R = 6.5 min.

8.8.9 6-Oxo-8,9,10,11,12,13,14,15-octahydro-*7H*-cycloundeca-[c][1]-benzopyran-3-*O*-sulphamate (125)

Compound **124** (300 mg; 1.05 mmol) was sulphamoylated by method 8.2.5 A, and the crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to

2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **125** as white fine crystals (133 mg; 35%). R_f: 0.37 ($CHCl_3$/ethyl acetate, 4:1); mp 145–148°C; MS (FAB^+) *m/z*: 731.2 [10, $(2M+H)^+$], 366.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 364.1 [100, $(M-H)^-$], 285.2 [40, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 366.1368, $C_{18}H_{24}NO_5S$ requires 366.1375; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.28-1.76 (m, 14H, 7×CH_2), 2.64 (t, 2H, C_8-CH_2, J = 7.0 Hz), 2.93 (t, 2H, C_{15}-CH_2, J = 7.0 Hz), 7.26 (dd, 1H, C_2-H, J = 2.1, 8.8 Hz), 7.29 (d, 1H, C_4-H, J = 2.1 Hz), 7.93 (d, 1H, C_1-H, J = 8.8 Hz) and 8.20 (s, 2H, NH_2); Found C, 59.20; H, 6.57; $C_{18}H_{23}NO_5S$ requires C, 59.16; H, 6.34%; HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 285.2 and 312.5 nm; t_R = 3.8 min.

8.8.10 Ethyl 2-oxocyclododecanecarboxylate (126)

Prepared by method 8.2.3, by stirring a mixture of NaH (2.19 g, 54.9 mmol), diethyl carbonate (100 ml) and cyclododecanone (5.0 g, 27 mmol). The crude dark yellow oily residue was purified by distillation under reduced pressure to give **126** as pale yellow oil (5.62 g, 81%). R_f: 0.72 (CH_2Cl_2); $bp_{0.23}$ 128–132°C; ([432]Lit. bp_3 155-157°C; MS (FAB^+) *m/z*: 255.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 253.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 255.1968, $C_{15}H_{27}O_3$ requires 255.1960; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.25 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 1.29-2.73 (m, 21H) and 4.16 (q, 2H, $CH_3C\underline{H}_2$, J = 6.4 Hz).

8.8.11 3-Hydroxy-6-oxo-7,8,9,10,11,12,13,14,15,16-decahydrocyclododeca-[c][1]-benzopyran (127)

Prepared by method 8.2.4, using resorcinol (1.08 g; 9.8 mmol), **126** (2.5 g; 9.8 mmol) and a mixture of CF_3COOH (1.5 ml; 20 mmol) and conc. H_2SO_4 (1.0 ml; 20 mmol). The crude pale yellow solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the pale yellow solid isolated was recrystallised from THF/hexane to give **127** white crystals (972 mg; 33%). R_f: 0.76 ($CHCl_3$/acetone, 3:1); mp 249-251°C; MS (FAB^+) *m/z*: 301.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 299.1 [100, $(M-H)^-$];

Acc. MS (FAB^+): 301.1806, $C_{19}H_{25}O_3$ requires 301.1804; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.39-2.89 (m, 16H, 8×CH_2), 2.93 (t, 2H, C_7-CH_2, J = 7.3 Hz), 3.22 (t, 2H, C_{16}-CH_2, J = 7.3 Hz), 6.66 (d, 1H, C_4-H, J = 2.3 Hz), 6.78 (dd, 1H, C_2-H, J = 2.3, 8.9 Hz), 7.63 (d, 1H, C_1-H, J = 8.9 Hz) and 10.77 (s, 1H, OH); Found C, 75.90; H, 8.03; $C_{19}H_{24}O_3$ requires C, 75.97; H, 8.05%; HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 324.4 nm; t_R = 4.2 min.

8.8.12 6-Oxo-7,8,9,10,11,12,13,14,15,16-decahydrocyclododeca-[c][1]-benzopyran-3-*O*-sulphamate (128)

Compound **127** (400 mg; 1.33 mmol) was sulphamoylated by method A (8.2.5) and the crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **128** as white fine crystals (182 mg; 36%). R_f: 0.47 ($CHCl_3$/ethyl acetate, 4:1); mp 173-175°C; MS (FAB^+) *m/z*: 380.1 [100, $(M+H)^+$], 301.1 [15, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 378.1 [100, $(M-H)^-$], 299.1 [50, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 380.1541, $C_{19}H_{26}NO_5S$ requires 380.1532; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.41-2.51 (m, 16H, 8×CH_2), 2.62 (t, 2H, C_7-CH_2, J = 7.3 Hz), 3.68 (t, 2H, C_{16}-CH_2, J = 7.6 Hz), 7.26 (dd, 1H, C_2-H, J = 2.4, 8.5 Hz), 7.28 (d, 1H, C_4-H, J = 2.4 Hz), 7.94 (d, 1H, C_1-H, J = 8.5 Hz) and 8.20 (s, 2H, NH_2); Found C, 60.30; H, 6.85; N, 3.62; $C_{19}H_{25}N\,O_5S$ requires C, 60.14; H, 6.64; N, 3.69%; HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 285.2 and 312.5 nm; t_R = 5.3 min.

8.8.13 Ethyl 2-oxocyclopentadecanecarboxylate (129)

Prepared by method 8.2.3, by stirring a mixture of NaH (891 mg, 22.3 mmol), diethyl carbonate (70 ml) and cycloundecanone (2.5g, 11 mmol). The crude yellow syrup was purified by flash chromatography (CH_2Cl_2) to give **129** as pale yellow oil (1.62 g, 49%). ([432]Lit. bp_1 168-169°C); R_f: 0.70 (CH_2Cl_2); MS (FAB^+) *m/z*: 297.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 295.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 297.2430, $C_{18}H_{33}O_3$ requires 297.2429; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.64 (t, 3H, $C\underline{H}_3CH_2$, J = 6.7 Hz),

1.15-1.62 (m, 25H), 2.55 (t, 2H, ring C_2-CH_2, J = 7.0 Hz) and 4.16 (q, 2H, $CH_3C\underline{H}_2$, J = 7.3 Hz).

8.8.14 3-Hydroxy-6-oxo-8,9,10,11,12,13,14,15,16,17,18,19-dodecahydro-*7H*-cyclopentadeca-[c][1]-benzopyran (130)

Prepared by method 8.2.4, using resorcinol (558 mg; 5.06 mmol), **129** (1.5 g; 5.1 mmol) and a mixture of CF_3COOH (1.0 ml; 10 mmol) and conc. H_2SO_4 (1.0 ml; 10 mmol). The crude brown solid was purified by flash chromatography ($CHCl_3$/acetone, 8:1 to 4:1 gradient) and the yellow solid isolated was recrystallised from THF/hexane to give **130** as pale yellow crystals (432 g; 25%). R_f: 0.69 ($CHCl_3$/acetone, 3:1); mp 209-211°C; MS (FAB^+) *m/z*: 343.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 341.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 343.2269, $C_{22}H_{31}O_3$ requires 343.2273; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.25-1.62 (m, 22H, 11×CH_2), 2.57 (t, 2H, C_7-CH_2, J = 7.8 Hz), 2.74 (t, 2H, C_{19}-CH_2, J = 7.0 Hz), 6.04 (s, 1H, OH), 6.81 (dd, 1H, C_2-H, J = 2.7, 8.9 Hz), 6.92 (d, 1H, C_4-H, J = 2.7 Hz) and 7.45 (d, 1H, C_1-H, J = 8.9 Hz); Found C, 77.12; H, 8.89; $C_{22}H_{30}O_3$ requires C, 77.16; H, 8.83%; HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 324.4 nm; t_R = 8.5 min.

8.8.15 6-Oxo-8,9,10,11,12,13,14,15,16,17,18,19-dodecahydrohydro-*7H*-cyclopentadeca-[c][1]-benzopyran-3-*O*-sulphamate (131)

Compound **130** (350 mg; 1.02 mmol) was sulphamoylated by method 8.2.5 A, and the crude white solid was purified by flash chromatography ($CHCl_3$/ethyl acetate, 8:1 to 2:1 gradient) to give a thick waxy solid, which was difficult to recrystallise. Further purification by preparative TLC ($CHCl_3$/ethyl acetate, 4:1) gave a white solid (201 mg), which was recrystallised from THF/hexane to give **131** as fine white film (185 mg; 43%). R_f: 0.50 ($CHCl_3$/ethylacetate, 4:1); mp 163-166°C; MS (FAB^+) *m/z*: 842.3 [70, $(2M+H)^+$], 422.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 841.4 [80, $(2M-H)^-$], 420.2 [100, $(M-H)^-$], 341.2 [60, $(M-H_2NSO_2)^-$]; Acc. MS (FAB^+): 422.1994, $C_{22}H_{32}NO_5S$ requires 422.1999; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.32-1.59 (m, 22H, 11×CH_2), 2.51-2.81

(m, 4H, C_7-CH_2 and C_{19}-CH_2), 7.26-7.28 (m, 2H, C_2-H and C_4-H), 7.89 (d, 1H, C_1-H, *J* = 7.8 Hz) and 8.19 (s, 2H, NH_2); Found C, 62.80; H, 7.56; N, 3.00; $C_{22}H_{31}NO_5S$ requires C, 62.68; H, 7.41; N, 3.32%; HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 285.2 and 313.7 nm; t_R = 4.2 min.

8.9 Indole Sulphamates

8.9.1 Indole-4-*O*-sulphamate (132)

Upon sulphamoylation by method 8.2.5 A, 4-hydroxyindole (200 mg; 1.5 mmol) gave a crude brown residue, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) to give **132** as a brown syrup (125 mg; 39%). R_f: 0.33 (ethyl acetate/hexane, 1:1); νmax (KBr) cm^{-1}: 3402 (N-H), 3071 (NH_2), 1396 (SO_2), 1100 (SO_2); MS (FAB^+) *m/z*: 425.1 [10, $(2M+H)^+$], 213.1 [100, $(M+H)^+$], 132.1 [35, $(M-H_2NSO_2)^+$]; MS (FAB^-) *m/z*: 423.1 [10, $(2M-H)^-$], 211.1 [100, $(M-H)^+$]; Acc. MS (FAB^+): 213.0321, $C_8H_9N_2O_3S$ requires 213.0334; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 6.48 (d, 1H, C_3-H, *J* = 3.9 Hz), 6.89 (d, 1H, C_5-H, *J* = 8.2 Hz), 7.07 (t, 1H, C_6-H, *J* = 7.8 Hz), 7.28-7.32 (m, 2H, C_2-H and C_7-H), 7.84 (s, 2H, NH_2) and 11.03 (s, 1H, NH).

8.9.2 Indole-5-*O*-sulphamate (133)

Upon sulphamoylation by method 8.2.5 A, 5-hydroxyindole (500 mg; 3.75 mmol) gave a crude brown solid, which was fractionated by flash chromatography (ethyl acetate/hexane gradient, 6:1 to 2:1) and the brown solid isolated was recrystallised from ethyl acetate/hexane to give **133** as brown crystals (256 mg; 32%). R_f: 0.48 (ethyl acetate/hexane, 1:1); mp 99-101°C; νmax (KBr) cm^{-1}: 3389 (N-H), 3220 (NH_2), 1390 (SO_2), 1091-1126 (SO_2); MS (FAB^+) *m/z*: 425.1 [30, $(2M+H)^+$], 213.1 [100, $(M+H)^+$], 132.1 [50, $(M-H_2NSO_2)^+$]; MS (FAB^-) *m/z*: 423.1 [10, $(2M-H)^-$], 211.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 213.0339, $C_8H_9N_2O_3S$ requires 213.0334; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 6.47 (m, 1H, C_3-H), 7.01 (dd, 1H, C_6-H, *J* = 2.3, 8.9 Hz), 7.40-7.45 (m, 3H, C_2-H, C_4-H and C_7-H), 7.76 (s, 2H, NH_2) and 11.25 (s, 1H, NH).

8.9.3 2-Carboxyindole-5-*O*-sulphamate (134)

Upon sulphamoylation by method 8.2.5 A, 5-hydroxy-2-indolecarboxylic acid (900 mg; 5.08 mmol) gave a crude white solid, which was purified by recrystallisation from ethyl acetate/hexane to give **134** as off-white crystals (642 mg; 49%). R_f: 0.64 (methanol); mp 203-205°C; νmax (KBr) cm^{-1}: 3382 (N-H), 2362-3331 (NH_2 and COOH), 1669 (C=O), 1393 (SO_2), 1085-1130 (SO_2); MS (FAB^+) *m/z*: 513.1 [10, $(2M+H)^+$], 257.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 511.1 [20, $(2M-H)^-$], 255.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 257.0224, $C_9H_9N_2O_5S$ requires 257.0232; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 7.09 (s, 1H, C_3-H), 7.13 (dd, 1H, C_6-H, J = 1.9, 8.9 Hz), 7.43 (d, 1H, C_7-H, J = 8.9 Hz), 7.52 (d, 1H, C_4-H, J = 2.3 Hz), 7.79 (s, 2H, NH_2), 7.84 (s, 1H, COOH) and 11.87 (s, 1H, NH); Found C, 42.40; H, 3.21; N, 10.80; $C_9H_8N_2O_5S$ requires C, 42.19; H, 3.15; N, 10.93%.

8.9.4 3-Methyl-1-pivaloylindole (135)

To a suspension of NaH (4.57 g; 114 mmol) in DMF (200 ml) was added 3-methylindole (10 g; 76 mmol) over 30 min at 0°C and stirred for 15 min. To this mixture was added pivaloyl chloride (12.2 ml; 99.1 mmol) dropwise at 4°C and stirred for further 15 min. This mixture was added to ice-H_2O (500 ml) and toluene (300 ml) and the organic layer was extracted. The combined extracts were washed with brine (3×200 ml), dried and evaporated to get a crude brown suspension, which was fractionated by flash chromatography ($CHCl_3$/hexane, 8:4 to 2:1 gradient) to give **135** as a pale yellow oil (12.7 g; 77%). R_f: 0.76 ($CHCl_3$/hexane, 3:2); νmax (KBr) cm^{-1}: 1689 (C=O); MS (FAB^+) *m/z*: 216.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 214.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 216.1713, $C_{14}H_{18}NO$ requires 216.1715; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.49 (s, 9H, $(CH_3)_3$), 2.29 (s, 3H, CH_3), 7.22-7.36 (m, 4H, C_4-H, C_5-H, C_6-H and C_7-H) and 7.48 (s, 1H, C_2-H); Found C, 78.04; H, 7.81; N, 6.43; $C_{14}H_{17}NO$ requires C, 78.10; H, 7.96; N, 6.51%.

8.9.5 6-Chloroacetyl-3-methyl-1-pivaloylindole (136)

To a suspension of $AlCl_3$ (28 g; 209 mmol) in 1,2-dichloroethane (250 ml) was added chloroacetyl chloride (17.5 ml; 218 mmol) dropwise at 0°C and stirred for 20 min and then warmed to 20°C. To this solution was added **135** (10 g; 47 mmol) dropwise over 3 h. The resulting mixture was stirred for an additional 15 min and then poured into ice-H_2O (500 ml). The organic layer was extracted with 1,2-dichloroethane (3×200 ml). The combined extracts were washed with water (3×500 ml), 5% $NaHCO_3$ (3×300 ml), dried and evaporated to get a crude brown solid, which was purified by recrystallistion from hot ethyl acetate to give **136** as pale brown crystals (6.48 g; 48%). R_f: 0.74 (ethyl acetate/hexane, 1:1); mp 121-123°C; νmax (KBr) cm^{-1}: 1690 (C=O); MS (FAB^+) *m/z*: 292.2 [100, $(M(^{35}Cl)+H)^+$]; MS (FAB^-) *m/z*: 290.1 [100, $(M(^{35}Cl)-H)^-$]; Acc. MS (FAB^+) *m/z*: 292.1103, $C_{16}H_{19}{}^{35}ClNO_2$ requires 292.1104 and 294.1082, $C_{16}H_{19}{}^{37}ClNO_2$ requires 294.1035; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.53 (s, 9H, $(CH_3)_3$), 2.33 (s, 3H, CH_3), 4.85 (s, 2H, CH_2Cl), 7.58 (d, 1H, C_4-H, J = 8.2 Hz), 7.68-7.69 (m, 1H), 7.95-7.97 (m, 1H) and 9.13 (s, 1H, C_2-H).

8.9.6 6-Chloroacetoxy-3-methyl-1-pivaloylindole (137)

To a suspension of anhydrous Na_2HPO_4 (11.7 g; 82.5 mmol) and **136** (6.0 g; 21 mmol) in CH_2Cl_2 (50 ml) was added 80% *m*-CPBA (5.3 g; 31 mmol) at 20°C and stirred for 1 h. The resulting mixture was poured into ice-H_2O (200 ml) and the organic layer was extracted with CH_2Cl_2 (3×100 ml). The combined extracts were washed with water (3×300 ml), 5% $NaHCO_3$ (3×200 ml), dried and evaporated to get a crude pale yellow syrup, which was fractionated by flash chromatography (ethyl acetate/hexane, 1:1) to give **137** as a pale yellow syrup (4.82 g; 72%). R_f: 0.87 (ethyl acetate/hexane, 1:1); νmax (KBr) cm^{-1}: 1695 (C=O); MS (FAB^+) *m/z*: 308.2 [100, $(M(^{35}Cl)+H)^+$]; MS (FAB^-) *m/z*: 306.2 [100, $(M(^{35}Cl)-H)^-$]; Acc. MS (FAB^+) *m/z*: 308.0978, $C_{16}H_{19}{}^{35}ClNO_3$ requires 307.0975 and 310.0983, $C_{16}H_{19}{}^{37}ClNO_3$ requires 309.0946; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.49 (s, 9H, $(CH_3)_3$), 2.28 (s, 3H, CH_3), 4.31 (s, 2H, CH_2Cl), 7.05

(dd, 1H, C_5-H, J = 2.3, 8.6 Hz), 7.46 (d, 1H, C_4-H, J = 8.6 Hz), 7.50 (d, 1H, C_7-H, J = 2.3 Hz) and 8.31 (s, 1H, C_2-H).

8.9.7 6-Hydroxy-3-methyl-1-pivaloylindole (138)

To a solution of **137** (3.5 g; 11 mmol) in methanol (100 ml) was added 15% sodium thiomethoxide (11 ml) at 20°C. After stirring for 2 h, the mixture was dissolved in ethyl acetate, neutralised with aq. 1M HCl and the organic layer was extracted with ethyl acetate (3×100 ml). The combined extracts were washed with water (3×200 ml), brine (3×100 ml), dried and evaporated to give a crude pale yellow solid, which was fractionated by flash chromatography (ethyl acetate/hexane, 8:1 to 6:1 gradient). The crude pale yellow solid was recrystallised from THF/hexane to give **138** as pale yellow crystals (78 mg; 3%). R_f: 0.82 (ethyl acetate/hexane, 1:1); mp 119-120°C; νmax (KBr) cm^{-1}: 3381 (O-H), 1653 (C=O); MS (FAB^+) *m/z*: 232.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 230.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 232.1323, $C_{14}H_{18}NO_2$ requires 232.1338; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.51 (s, 9H, $(CH_3)_3$), 2.25 (s, 3H, CH_3), 6.05 (s, 1H, OH), 6.89 (dd, 1H, C_5-H, J = 2.3, 8.2 Hz), 7.32 (d, 1H, C_4-H, J = 8.2 Hz), 7.37 (d, 1H, C_7-H, J = 2.4 Hz) and 8.18 (s, 1H, C_2-H); Found C, 72.61; H, 7.29; N, 5.88; $C_{14}H_{17}NO_2$ requires C, 72.70; H, 7.41; N, 6.06%.

8.9.8 3-Methyl-1-pivaloylindole-6-*O*-sulphamate (139)

Upon sulphamoylation by method 8.2.5 A, compound **138** (50 mg; 0.22 mmol) gave a crude brown residue, which was purified by preparative TLC to give **139** as an off white syrup solidified on standing (16 mg; 24%). R_f: 0.51 (ethyl acetate/hexane, 1:1); mp 129-132°C; νmax (KBr) cm^{-1}: 3387 (NH_2), 1649 (C=O), 1399 (SO_2), 1101-1129 (SO_2); MS (FAB^+) *m/z*: 311.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 309.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 311.1087, $C_{14}H_{19}N_2O_4S$ requires 311.1066; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.41 (s, 9H, $(CH_3)_3$), 2.22 (s, 3H, CH_3), 6.85 (dd, 1H, C_5-H, J = 2.4, 8.2 Hz), 7.41 (d, 1H, C_4-H, J = 8.2 Hz), 7.36 (d, 1H, C_7-H, J = 2.4 Hz), 7.57 (s, 2H, NH_2) and 8.06 (s, 1H, C_2-H).

8.9.9 5-Hydroxy-2-methylindole (140)

To a suspension of aluminium chloride (2.48 g; 18.6 mmol) in dry toluene (80 ml), 5-methoxy-2-methylindole (1.0 g; 6.2 mmol) was added. The yellow mixture was boiled under reflux for 3 h under N_2. Upon cooling of the brown/green mixture to 0°C, water (50 ml) was added cautiously and the organic fraction was extracted into ethyl acetate (3×100 ml), washed with water (3×100 ml), dried, filtered and evaporated to give a crude brown residue, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 1:1 gradient). The brown solid isolated was recrystallised from THF/hexane to give **140** as a pale brown crystals (321 mg; 35%). R_f : 0.51 (ethyl acetate/hexane, 1:1); mp 128-129°C ([433]Lit. mp 132-134°C); νmax (KBr) cm^{-1}: 3883 (O-H), 3393 (N-H); MS (FAB^+) *m/z*: 148.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 146.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 148.0797, $C_9H_{10}NO$ requires 148.0762; ^1H NMR (400 MHz; acetone-d_6) δ_H: 2.36 (s, 3H, CH_3), 5.97 (s, 1H, C_3-H), 6.58 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz) 6.84 (broad s, 1H, C_4-H), 7.08 (d, 1H, C_7-H, J = 8.2 Hz), 7.51 (s, 1H, OH) and 9.69 (s, 1H, NH); Found C, 73.45; H, 6.17; N, 9.50; C_9H_9NO requires C, 73.45; H, 6.16; N, 9.52%.

8.9.10 3-Aminosulphonyl-5-sulphamoyloxy-2-methylindole (141)

Upon sulphamoylation by method 8.2.5 A compound **140** (200 mg; 1.36 mmol) gave a crude white solid, which was fractionated by flash chromatography ($CHCl_3$/methanol, 6:1 to 1:1 gradient). The pale brown solid isolated was recrystallised from THF/hexane to give **141** as a pale brown crystals (72 mg; 17%). R_f: 0.70 ($CHCl_3$/methanol, 3:2); mp 230-233°C; νmax (KBr) cm^{-1}: 3336 (N-H), 3214-3262 (NH_2), 1379 (SO_2), 1080 (SO_2), MS (FAB^+) *m/z*: 306.0 [100, $(M+H)^+$], 226.1 [40, $(M-H_2NSO_2)^+$]; MS (FAB^-) *m/z*: 304.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 306.0209, $C_9H_{12}N_3O_5S_2$ requires 306.0218; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.48 (s, 3H, CH_3), 7.04 (dd, 1H, C_6-H, J = 2.3, 8.9 Hz), 7.37 (d, 1H, C_7-H, J = 8.6 Hz), 7.69 (d, 1H, C_4-H, J = 2.3 Hz), 7.84 (s, 4H, NH_2) and 11.86 (s, 1H, NH).

8.9.11 1,2-Dimethyl-5-methoxyindole (142)

A solution of 5-methoxy-2-methylindole (3.0 g; 19 mmol) in anhy. DMF (100 ml) was treated with NaH (1.11 g; 27.9 mmol) under N_2 and stirred until all of the H_2 evolution had ceased. Methyl iodide (1.75 ml; 27.9 mmol) was introduced and stirred for 3 h. The mixture was poured into brine (200 ml) and the organic layer was extracted with ethyl acetate (3×100 ml). The combined extracts were washed with water (3×200 ml), dried and evaporated to give a crude brown solid, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The brown solid isolated (1.63 g) was recrystallised from ethyl acetate/hexane to give **142** as a pale yellow needles (721 mg; 22%). R_f: 0.76 (ethyl acetate/hexane, 1:1); mp 74-76°C ([434]Lit. mp 73–74°C); MS (FAB^+) *m/z*: 176.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 174.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 176.1003, $C_{11}H_{13}NO$ requires 176.0997; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.39 (s, 3H, CH_3), 3.63 (s, 3H, NCH_3), 3.84 (s, 3H, OCH_3), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.9 Hz), 7.01 (d, 1H, C_4-H, J = 2.3 Hz) and 7.13 (d, 1H, C_7-H, J = 8.9 Hz); Found C, 75.40; H, 7.54; N, 7.90; $C_{11}H_{13}NO$ requires C, 75.40; H, 7.48; N, 7.99%.

8.9.12 1,2-Dimethyl-5-hydroxyindole (143)

Using the procedure described for the synthesis of compound **140**, demethylation of compound **142** (1.0 g; 5.7 mmol) gave a crude brown solid, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The pale orange solid isolated was recrystallised from ethyl acetate/hexane to give **143** as a pale yellow crystals (514 mg; 56%). R_f: 0.72 (ethyl acetate/hexane, 1:1); mp 148-149°C ([435]Lit. mp 143°C); MS (FAB^+) *m/z*: 162.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 160.2 [100, $(M-H)^-$], 145.1 [60, $(M-H-CH_3)^-$]; Acc. MS (FAB^+): 162.0967, $C_{10}H_{12}NO$ requires 162.0919; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.39 (s, 3H, CH_3), 3.62 (s, 3H, NCH_3), 4.79 (s, 1H, OH), 6.18 (s, 1H, C_3-H), 6.71 (dd, 1H, C_6-H, J = 2.7, 8.6 Hz), 6.93 (d, 1H, C_4-H, J = 2.3 Hz) and 7.69 (d, 1H, C_7-H, J = 8.6 Hz); Found C, 74.60; H, 6.94; N, 8.68; $C_{10}H_{11}NO$ requires C, 74.51; H, 6.88; N, 8.69%.

8.9.13 1,2-Dimethylindole-5-*O*-sulphamate (144)

Upon sulphamoylation by method 8.2.5 A, compound **143** (400 mg; 2.48 mmol) gave a crude orange residue, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The yellow solid isolated was recrystallised from ethyl acetate/hexane to give **144** as fine grey crystals (81 mg; 14%). R_f: 0.47 (ethyl acetate/hexane, 1:1); mp 163-165°C; νmax (KBr) cm^{-1}: 3283-3387 (NH_2), 1356 (SO_2), 1100-1138 (SO_2); MS (FAB^+) *m/z*: 241.1 [100, $(M+H)^+$], 160 ($M-H_2NSO_2$); MS (FAB^-) *m/z*: 239.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 241.0641, $C_{10}H_{13}N_2O_3S$ requires 241.0647; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 2.40 (s, 3H, CH_3), 3.67 (s, 3H, NCH_3), 6.25 (s, 1H, C_3-H), 6.97 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.31 (d, 1H, C_4-H, J = 2.3 Hz), 7.39 (d, 1H, C_7-H, J = 8.6 Hz) and 7.75 (s, 2H, NH_2); Found C, 49.90; H, 5.09; N, 11.57; $C_{10}H_{12}N_2O_3S$ requires C, 49.99; H, 5.03; N, 11.66%.

8.9.14 1-Ethyl-2-methyl-5-methoxyindole (145)

Following the method described for compound **142**, a DMF (100 ml) solution of 5-methoxy-2-methylindole (2.5 g; 16 mmol) was treated with NaH (931 mg; 23.3 mmol) and ethyl iodide (1.7 ml; 23 mmol). The crude brown syrup isolated was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 1:1 gradient) to give **145** as a brown thick syrup (1.98 g; 68%). R_f: 0.77 (ethyl acetate/hexane, 1:1); [436]Lit. mp 21°C; νmax (KBr) cm^{-1}: 2934 (OCH_3); MS (FAB^+) *m/z*: 189.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 188.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 189.1672, $C_{12}H_{16}NO$ requires 189.1699; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.27 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 2.36 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 4.02 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 6.13 (s, 1H, C_3-H), 6.78 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 6.99 (d, 1H, C_4-H, J = 2.3 Hz) and 7.11 (d, 1H, C_7-H, J = 8.9 Hz); Found C, 76.11; H, 7.72; N, 7.47; $C_{12}H_{15}NO$ requires C, 76.16; H, 7.99; N, 7.40%.

8.9.15 1-Ethyl-2-methyl-5-hydroxyindole (146)

Following the procedure described for the preparation of compound **140**, demethylation of compound **145** (1.5 g; 7.9 mmol) gave a crude brown solid, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The yellow solid isolated was recrystallised from ethyl acetate/hexane to give **146** as pale yellow crystals (721 mg; 52%). R_f: 0.73 (ethyl acetate/hexane, 1:1); mp 109-111°C ([436]Lit. mp 114–115°C); νmax (KBr) cm^{-1}: 3267 (O-H); MS (FAB^+) *m/z*: 176.1 [100, $(M+H)^+$]; Acc. MS (FAB^+): 176.1059, $C_{11}H_{14}NO$ requires 176.1075; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 1.31 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 2.39 (s, 3H, CH_3), 4.07 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 4.82 (s, 1H, OH), 6.12 (s, 1H, C_3-H), 6.71 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 6.92 (d, 1H, C_4-H, J = 2.7 Hz) and 7.11 (d, 1H, C_7-H, J = 8.6 Hz); Found C, 75.60; H, 7.44; N, 7.92; $C_{11}H_{13}NO$ requires C, 75.40; H, 7.48; N, 7.99%.

8.9.16 1-Ethyl-2-methylindole-5-*O*-sulphamate (147)

Upon sulphamoylation by method 8.2.5 A, compound **146** (400 mg; 2.28 mmol) gave a crude brown solid, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The pale yellow solid isolated was recrystallised from ethyl acetate/hexane to give **147** as fine pale yellow crystals (56 mg; 10%). R_f: 0.53 (ethyl acetate/hexane, 1:1); mp 116-117°C; νmax (KBr) cm^{-1}: 3255–3327 (NH_2), 1351 (SO_2), 1100-1143 (SO_2); MS (FAB^+) *m/z*: 254.1 [100, $(M+H)^+$], 174.1 [55, $(M+H-HNSO_2)^+$]; MS (FAB^-) *m/z*: 253.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 254.0817, $C_{11}H_{15}N_2O_3S$ requires 254.0806; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 1.22 (t, 3H, $CH_2C\underline{H}_3$, J = 7.0 Hz), 2.41 (s, 3H, CH_3), 4.16 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 6.24 (s, 1H, C_3-H), 6.97 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.32 (d, 1H, C_4-H, J = 2.3 Hz), 7.41 (d, 1H, C_7-H, J = 8.9 Hz) and 7.73 (s, 2H, NH_2); Found C, 51.70; H, 5.43; N, 11.00; $C_{11}H_{14}N_2O_3S$ requires C, 51.95; H, 5.55; N, 11.02%.

8.9.17 1,2-Dimethyl-3-benzoyl-5-methoxyindole (148)

To a solution of $AlCl_3$ (3.8 g; 28.5 mmol) in CH_2Cl_2 (40 ml) at 25°C was added benzoyl chloride (5 ml; 42.8 mmol) and stirred for 15 min. When a clear mixture resulted, a solution of **142** (2.5 g; 14.27 mmol) in CH_2Cl_2 (10 ml) was added dropwise and stirred for 3 h. The mixture was poured into crushed ice and the organic layer was extracted with CH_2Cl_2 (3×100 ml). The combined extracts were washed with water (3×200 ml), brine (3×100 ml), aq. $NaHCO_3$ (2×50 ml), dried and evaporated to give a crude brown residue, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 1:1 gradient). The pale brown solid isolated was recrystallised from THF/hexane to give **148** as pale brown crystals (532 mg; 13%). R_f: 0.59 (ethyl acetate/hexane, 1:1); mp 104-107°C; νmax (KBr) cm^{-1}: 2936 (OCH_3), 1624 (C=O); MS (FAB^+) *m/z*: 559.2 [10, $(2M+H)^+$], 280.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 278.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 280.1332, $C_{18}H_{18}NO_2$ requires 280.1337; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.53 (s, 3H, CH_3), 3.66 (s, 3H, NCH_3), 3.71 (s, 3H, OCH_3), 6.83 (d, 1H, C_4-H, J = 2.3 Hz), 7.20 (dd, 1H, C_6-H, J = 2.3, 8.6 Hz), 7.46 (d, 2H, Ph-H, J = 7.4 Hz), 7.53 (d, 2H, Ph-H, J = 7.4 Hz) and 7.73 (d, 1H, C_7-H, J = 8.9 Hz); Found C, 77.60; H, 6.09; N, 4.81; $C_{18}H_{17}NO_2$ requires C, 77.40; H, 6.13; N, 5.01%.

8.9.18 1,2-Dimethyl-3-benzoyl-5-hydroxyindole (149)

Using the procedure described for the preparation of compound **140**, demethylation of compound **148** (400 mg; 1.43 mmol) gave a crude brown solid, which was fractionated by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The brown solid isolated was recrystallised from THF/hexane to give **149** as fine brown crystals (167 mg; 44%). R_f: 0.41 (ethyl acetate/hexane, 1:1); mp 242-245°C ([437]Lit. mp 272–275°C); νmax (KBr) cm^{-1}: 3156 (O-H), 1621 (C=O); MS (FAB^+) *m/z*: 266.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 264.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 266.1174, $C_{17}H_{16}NO_2$ requires 266.1181; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.51 (s, 3H, CH_3), 3.49 (s, 1H, OH), 3.66 (s, 3H, NCH_3), 6.79 (dd, 1H, C_6-H, J = 2.3, 8.9 Hz), 7.18 (d, 1H, C_4-H, J = 2.3 Hz),

7.54 (d, 1H, C_7-H, J = 8.8 Hz) and 7.71-7.75 (m, 5H, Ph-H); Found C, 76.92; H, 5.55; N, 4.98; $C_{17}H_{15}NO_2$ requires C, 76.96; H, 5.70; N, 5.28%.

8.9.19 1,2-Dimethyl-3-benzoylindole-5-*O*-sulphamate (150)

Upon sulphamoylation by method 8.2.5 A, compound **149** (100 mg; 0.38 mmol) gave a crude brown solid, which was purified by preparative TLC to give **150** as brown wax, which solidified on standing (42 mg; 32%). R_f: 0.44 (ethyl acetate/hexane, 1:1); mp 63-64°C; νmax (KBr) cm^{-1}: 3306 (NH_2), 1609 (C=O), 1379 (SO_2), 1082-1122 (SO_2); MS (FAB^+) *m/z*: 345.1 [100, $(M+H)^+$], 264.1 [15, $(M-H_2NSO_2)^+$]; MS (FAB^-) *m/z*: 343.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 345.0908, $C_{17}H_{17}O_4N_2S$ requires 345.0909; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 2.39 (s, 3H, CH_3), 3.63 (s, 3H, NCH_3), 6.78 (dd, 1H, C_6-H, J = 2.1, 8.6 Hz), 7.22 (d, 1H, C_4-H, J = 2.1 Hz), 7.57 (d, 1H, C_7-H, J = 8.9 Hz), 7.69-7.73 (m, 5H, Ph-H) and 7.78 (s, 2H, NH_2).

8.10 Derivatives of estrone-3-sulphonamides and *S*-sulphamates

8.10.1 3-[(*N,N*-Dimethylthiocarbamoyl)oxy]estra-1,3,5(10)-trien-17-one (151)

NaH (60% dispersion in mineral oil; 888 mg; 22.2 mmol) was added to a solution of estrone (5.0 g; 18 mmol) in dry DMF (75 ml) under N_2. After the evolution of H_2 had ceased, the solution was cooled in an ice bath and *N,N*-dimethylthiocarbamoyl chloride (3.43 g; 27.8 mmol) was added. The resulting solution was heated at 80°C for 1 h. Cooled to R.T and poured into cold water (100 ml). The crude pale brown precipitate was filtered, dried under vacuum and purified by flash chromatography (CH_2Cl_2). The white solid isolated was recrystallised from ethyl acetate to give **151** as fine white crystals (5.29 g; 80%). R_f: 0.52 (CH_2Cl_2); mp 211-213°C ([184]Lit. mp 210-212°C); MS (FAB^+) *m/z*: 358.2 [100, $(M+H)^+$]; Acc. MS (FAB^+): 358.1767, $C_{21}H_{28}NO_2S$ requires 358.1762; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.26-2.54 (m, 13H), 2.91-2.98 (m, 2H, CH_2), 3.33 (s, 3H, NCH_3), 3.46 (s, 3H, NCH_3), 6.79 (d, 1H, C_4-H, J = 2.3 Hz), 6.86 (dd, 1H, C_2-H, J = 2.7, 8.6 Hz) and 7.30 (d, 1H, C_1-H, J = 8.2 Hz);

HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 249.8 and 324.4 nm; t_R = 10.7 min (c.f. for estrone = 4.4 min).

8.10.2 3-[(*N,N*-Dimethylcarbamoyl)thio]estra-1,3,5(10)-trien-17-one (152)

A suspension of **151** (4.0 g; 11 mmol) in white heavy mineral oil (70 ml) was heated under N_2 at 250°C for 6 h. The mixture was cooled to R.T. The precipitate formed was diluted with petroleum ether (40-60°C) (50 ml), filtered, washed thoroughly with petroleum ether and hexane and dried under vacuum. The crude pale brown solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the off white solid isolated was recrystallised from hot ethyl acetate to give **152** as off white needles (3.02 g, 76%). R_f: 0.56 (hexane/ethyl acetate, 1:1); mp 182-184°C ([184]Lit. mp 173-175°C); MS (FAB^+) *m/z*: 358.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 358.1771, $C_{21}H_{28}NO_2S$ requires 358.1763; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.24-2.54 (m, 13H), 2.91-2.94 (m, 2H, CH_2), 3.03 (s, 3H, NCH_3), 3.89 (s, 3H, NCH_3), 7.21-7.23 (m, 1H, Ar-H), 7.24-7.26 (m, 1H, Ar-H) and 7.28-7.29 (m, 1H, Ar-H); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 367 nm; t_R = 14.1 min.

8.10.3 3-Mercaptoestra-1,3,5(10)-trien-17-one (153)

To a solution of **152** (2.5 g; 7.0 mmol.) in absolute ethanol (200 ml) was added NaOH (7.84 g; 196 mmol) dissolved in water (30 ml) and refluxed under boiling for 4 h under N_2. The mixture was cooled to R.T and the excess NaOH was neutralised with 8% HCl (200 ml). This was then concentrated to remove ethanol and poured into water. The precipitate formed was filtered, washed with water and dried. The crude pale brown solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the off-white solid isolated was recrystallised from hot ethyl acetate to give **153** as off-white crystals (1.72 g; 86%). R_f: 0.91 (hexane/ethyl acetate, 1:1); mp 187-189°C ([184]Lit. mp 195-197°C); MS (FAB^+) *m/z*: 286.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 285.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 286.1386, $C_{18}H_{22}OS$ requires 286.1391); ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.23-2.55 (m, 13H), 2.85-2.88 (m, 2H, CH_2),

3.36 (s, 1H, SH exchanged with D_2O), 7.05 (d, 1H, C_4-H, J = 2.3 Hz), 7.07-7.11 (m, 1H, C_2-H) and 7.17 (d, 1H, C_1-H, J = 8.6 Hz); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 264.4 nm; t_R = 16.1 min.

8.10.4 Estra-1,3,5(10)-trien-17-one-3-*S*-sulphamate (154)

Upon sulphamoylation by method 8.2.5 A, compound **153** (100 mg; 0.56 mmol) gave a crude pale yellow solid, which was purified by flash chromatography (ethyl acetate/hexane, 8:1 to 2:1 gradient) and the yellow solid isolated (91 mg) was recrystallised from ethyl acetate/hexane to give a pale yellow crystals of **154** (72 mg; 56%). R_f: 0.74 (ethyl acetate/hexane, 4:1); mp 115-118°C ([194]Lit. mp 101-112°C); MS (e.s. in MeOH) *m/z*: 366.3 [60, $(M+H)^+$], 287.4 [30, $(M+H-HNSO_2)^+$], 97.2 [100, $H_2NSO_2+H)^+$]; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.90 (s, 3H, CH_3), 1.13-2.54 (m, 13H), 2.86-2.89 (m, 2H, CH_2), 5.14 (s, 2H, NH_2) and 7.24-7.28 (m, 3H, Ar-H); HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 256.9 and 271 nm; t_R = 10.2 min.

8.10.5 3-(Benzylthio)estra-1,3,5(10)-trien-17-one (155)

To a solution of **153** (1.5 g; 5.3 mmol) in dry DMF (70 ml) was added NaH (255 mg; 6.38 mmol) and stirred under N_2. After all the H_2 had evolved, benzyl bromide (0.8 ml; 6.38 mmol) was added and stirred for 4 h under N_2. The mixture was poured into cold water and the resulting precipitate was filtered, washed with water and dried to obtain a pale yellow solid. An analytical sample was purified by flash chromatography (ethyl acetate/hexane, 8:1 to 2:1 gradient) and the yellow solid isolated was recrystallised from ethyl acetate to give **155** as off-white crystals (1.62 g, 82%). R_f: 0.77 (hexane/ethyl acetate, 1:1); mp 122-125°C ([184]Lit. mp 127-129°C); MS (FAB^+) *m/z*: 376.0 [100, $(M+H)^+$], 91.1 [85, $(PhCH_2+H)^+$]; Acc. MS (FAB^+): 376.1853, $C_{25}H_{28}OS$ requires 376.1861; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.24-2.54 (m, 13H), 2.83-2.87 (m, 2H, CH_2), 4.09 (s, 2H, SCH_2), 7.05-7.07 (m, 1H, C_4-H), 7.10 (dd, 1H, C_2-H, J = 1.9, 8.2 Hz), 7.18 (d, 1H, C_1-H, J = 8.2 Hz) and 7.29-7.31 (m, 5H, Ph-H); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 257.4 nm; t_R = 8.3 min.

8.10.6 Estra-1,3,5(10)-trien-17-one-3-sulphonylchloride (156)

To a cool suspension of **155** (1.17 g; 3.05 mmol) in glacial acetic acid (50 ml) and water (2 ml), Cl_2 gas was bubbled for 15 min until completely reacted. Cold water (200 ml) was added and the mixture was extracted with ethyl acetate (200 ml). The combined organic layers were then washed well with 5% $NaHCO_3$, dried and evaporated to get a crude pale yellow solid, which was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The white solid isolated (987 mg) was recrystallised from ethyl acetate/hexane to give **156** as fine white crystals (832 mg, 78%). R_f: 0.62 (ethyl acetate/hexane, 1:1); mp 154-156°C ([184]Lit. mp 153-155°C); MS (FAB^+) *m/z*: 353.0 [100, $(M+H)^+$], 317.1 [60, $(M-^{35}Cl)^+$]; MS (FAB^-) *m/z*: 351.1 [100, $(M-H)^-$]; Acc. MS *m/z* (FAB^+): 352.0895, $C_{18}H_{21}{}^{35}ClO_3S$ requires 352.0899 and 354.0964, $C_{18}H_{21}{}^{37}ClO_3S$ requires 354.0970; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.93 (s, 3H, CH_3), 1.46-2.57 (m, 13H), 3.01-3.04 (m, 2H, CH_2), 7.53 (d, 1H, C_1-H, J = 8.6 Hz), 7.76 (d, 1H, C_4-H, J = 2.3 Hz) and 7.79 (dd, 1H, C_2-H, J = 2.3, 8.6 Hz); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 229.8 and 271 nm; t_R = 4.6 min.

8.10.7 Estra-1,3,5(10)-trien-17-one-3-sulphonylfluoride (157)

To a solution of **156** (200 mg; 0.57 mmol) in 1,4-dioxane (4 ml) was added potassium fluoride (60 mg; 1.0 mmol) in water (1 ml) and heated at 50-55°C for 24 h. The solvent was evaporated to get a crude white solid, which was purified by flash chromatography (ethyl acetate/hexane, 8:1 to 2:1 gradient). The white solid isolated was recrystallised from THF/hexane to give **157** as white crystals (114 mg, 60%). R_f: 0.48 (ethyl acetate/hexane, 1:1); mp 163-165°C ([184]Lit. mp 160-162°C); MS (FAB^+) *m/z*: 337.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 335.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 337.1195 $C_{18}H_{22}FO_3S$ requires 337.1183; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.93 (s, 3H, CH_3), 1.46-2.57 (m, 13H), 2.99-3.07 (m, 2H, CH_2), 7.53 (d, 1H, C_1-H, J = 8.2 Hz), 7.74 (d, 1H, C_4-H, J = 1.9 Hz) and 7.77 (dd, 1H, C_2-H, J = 1.9, 8.2 Hz); Found C, 64.28; H,

6.23; $C_{18}H_{21}FO_3S$ requires C, 64.26; H, 6.29%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 233.3 and 272.2 nm; t_R = 11.3 min.

8.10.8 Estra-1,3,5(10)-trien-17-one-3-sulphonamide (158)

Cold concentrated aqueous ammonium hydroxide (35% NH_3 solution; 7 ml) was added to a solution of **156** (200 mg; 0.57 mmol) in cold acetone (7 ml) and stirred at R.T. for 15 min. The solution was extracted from CH_2Cl_2 (30 ml) and the combined extracts were then washed with water and brine, dried and evaporated to get a crude white solid, which was purified by flash chromatography (ethyl acetate/hexane, 8:1 to 1:1 gradient). The white solid isolated was recrystallised from ethyl acetate/hexane to give **158** as fine white crystals (155 mg; 82%). R_f: 0.36 (ethyl acetate/hexane, 1:1); mp 232-234°C ([184]Lit. mp 228-230°C); MS (FAB^+) *m/z*: 334.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 332.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 334.1471, $C_{18}H_{24}NO_3S$ requires 334.1477; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.24-2.58 (m, 13H), 2.94-2.99 (m, 2H, CH_2), 5.46 (s, 2H, NH_2), 7.41 (d, 1H, C_1-H, J = 8.2 Hz), 7.66 (d, 1H, C_4-H, J = 1.9 Hz) and 7.68 (dd, 1H, C_2-H, J = 1.9, 8.2 Hz); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 228.6 and 269.8 nm; t_R = 2.3 min.

8.10.9 Estra-1,3,5(10)-trien-17-ol-3-sulphonamide (159)

To a solution of **158** (50 mg; 0.15 mmol) in isopropanol (3 ml) and THF (0.5 ml) was added $NaBH_4$ (8.5 mg; 0.2 mmol) at 0°C. The mixture was stirred for 30 min at 0°C and quenched with aqueous NH_4Cl (5 ml). H_2O (30 ml) was added and the white precipitate formed was filtered out, washed with water, dried under vacuum and purified by preparative TLC to give **159** as white fine powder (13 mg; 26%). R_f: 0.38 (ethyl acetate/hexane, 1:1); mp 191-193°C; MS (FAB^+) *m/z*: 336.2 [30, $(M+H)^+$], 97.1 [100, $(SO_2NH_2+H)^+$]; MS (FAB^-) *m/z*: 334.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 336.1545, $C_{18}H_{26}NO_3S$ requires 336.1555; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.67 (s, 3H, CH_3), 1.21-2.51 (m, 13H), 2.77-2.86 (m, 2H, CH_2), 3.53 (t, 1H, CH, J = 8.6 Hz), 4.54 (s, 1H, OH), 7.45 (s, 2H, NH_2) and 7.47-7.56 (m, 3H, Ar-H); Found C, 64.30; H,

7.41; N, 4.05; $C_{18}H_{25}NO_3S$ requires C, 64.45; H, 7.51, N, 4.18%; HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 223.1 and 277.1 nm; t_R = 1.8 min.

8.10.10 17,17-Ethylenedioxy-1,3,5[10]-estratriene-3-ol (160)

A suspension of estrone (25 g; 93 mmol), toluene (300 ml), ethylene glycol (26 ml; 462 mmol) and *p*-toluenesulphonic acid monohydrate (300 mg) were boiled under reflux for 14 h under Dean-Stark conditions. The resultant pale pink solution was poured onto saturated aq. $NaHCO_3$ (150 ml) and diluted with ethyl acetate (250 ml). The organic layers were extracted into ethyl acetate (100 ml), washed with water (3×350 ml) and brine (3×150 ml). The mixture was dried and evaporated to give a crude off-white crystalline solid (28.7 g, 96% crude). An analytical sample was purified by flash chromatography (ethyl acetate/hexane, 8:1 to 1:1 gradient) and the white solid isolated was recrystallised from ethyl acetate to give **160** as white fine crystals. R_f: 0.41 (ethyl acetate/hexane, 1:1); mp 173-175°C; ([438]Lit. mp 164-167°C); MS (FAB^+) *m/z*: 315.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 313.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 315.1964, $C_{20}H_{27}O_3$ requires 315.1960; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (s, 3H, CH_3), 1.30-2.18 (m, 11H), 2.21-2.30 (m, 1H, CH), 2.32-2.46 (m, 1H, CH), 2.77-2.87 (m, 2H, CH_2), 3.89-3.99 (m, 4H, OCH_2CH_2O), 4.71 (s, 1H, OH), 6.56 (d, 1H, C_4-H, J = 2.7 Hz), 6.62 (dd, 1H, C_2-H, J = 2.7, 8.6 Hz) and 7.15 (d, 1H, C_1-H, J = 8.2 Hz); ^{13}C NMR (100.5 MHz; $CDCl_3$) δ_C: 14.2 (CH_3), 22.4, 26.9, 32.1, 33.2 (all CH_2), 37.7, 43.6, 49.2 (all CH), 64.9, 65.1 (all CH_2), 110.2, 112.3 (both CH), 116.1 (C), 127.1 (CH), 130.6, 138.1 (all C) and 154.6 (COH); Found C, 76.70; H, 8.15; $C_{20}H_{26}O_3$ requires C, 76.40; H, 8.33%; HPLC: MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 279.3 and 341.1 nm; t_R = 12.3 min.

8.10.11 Methyl chloromethyl ether (161)

A mixture of dimethoxymethane (300 ml, 3.94 mmol; 1 eq.), decanoyl chloride (818 ml; 3.94 mmol; 1 eq.) and conc. H_2SO_4 (2.5 ml) was stirred at 50–55°C for 4 h. Na_2CO_3 (1 g) was added and **161** was collected by distillation under N_2 as a clear

liquid (228 ml, 72 %). bp 56-59°C ([439]Lit. bp 57°C); ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 3.52 (s, 3H, OCH_3) and 5.47 (s, 2H, CH_2Cl).

8.10.12 17,17-Ethylenedioxy-1,3,5[10]-estratriene-3-methoxymethyl ether (162)

NaH (4.77 g, 119 mmol) was added in portions to a 0°C solution of **160** (25 g, 80 mmol) dissolved in dry DMF (400 ml). After H_2 evolution had ceased **161** (1.82 ml; 159 mmol) was added dropwise and the stirred solution was allowed to come to R.T. overnight. The solution was then poured onto aq. ammonia solution (100 ml, 2M) to destroy any remnant haloether contaminants. Ethyl acetate (500 ml) was added and the organic layer was then washed with water and brine (5×200 ml), dried and evaporated. The crude pale yellow syrup was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **162** as a colourless oil, which solidified on standing to give a white crystalline solid (27.2 g, 95%). R_f: 0.74 (ethyl acetate/hexane, 1:1); mp 63–65°C ([388]Lit. mp 62-63°C); MS (FAB^+) *m/z*: 358.1 [100, $(M+H)^+$]; Acc. MS (FAB^+): 358.2158, $C_{22}H_{31}O_4$ requires 358.2144; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (s, 3H, CH_3), 1.25-2.05 (m, 11H), 2.21-2.29 (m, 1H, CH), 2.30-2.34 (m, 1H, CH), 2.83-2.86 (m, 2H, CH_2), 3.47 (s, 3H, OCH_3), 3.88-3.98 (m, 4H, OCH_2CH_2O), 5.14 (s, 2H, OCH_2O), 6.77 (d, 1H, C_4-H, J = 2.7 Hz), 6.82 (dd, 1H, C_2-H, J = 2.7, 8.6 Hz) and 7.22 (d, 1H, C_1-H, J = 8.6 Hz); ^{13}C NMR (100.5 MHz; $CDCl_3$) δ_C: 14.3 (CH_3), 22.3, 26.1, 26.9, 29.7, 30.7, 34.2 (all CH_2), 38.9, 43.7 (both CH), 46.1 (C), 49.3 (CH), 55.9 (CH_3), 64.5, 65.2, 94.5 (all CH_2), 113.7, 116.2 (both CH), 119.4 (C), 126.3 (CH) and 134.0, 138.1, 155.0 (all C); Found C, 72.90; H, 8.33; $C_{22}H_{30}O_4$ requires C, 73.71; H, 8.44%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 276.1 and 379 nm; t_R = 15.8 min.

8.10.13 2-Ethyl-17,17-ethylenedioxy-3-(methoxymethyloxy)-1,3,5[10]-estratriene (163)

A solution of **162** (10 g; 28 mmol) in freshly distilled THF (300 ml) was cooled to –78°C in a dry ice/acetone bath for 1 h and then treated with ^{sec}butyl lithium (7.0 ml; 84

mmol.) dropwise over 4 h. After stirring for further 4 h, ethyl iodide (9.0 ml; 112 mmol, freshly distilled from finely chopped sodium) was added and the mixture was allowed to warm to R.T. overnight. Saturated NH_4Cl solution (20 ml) was added followed by diethyl ether (400 ml). The mixture was washed with water (250 ml), brine (2×350 ml), dried and evaporated to give a white suspension, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **163** as colourless syrup (9.82 g, 91 %). R_f: 0.84 (ethyl acetate/hexane, 1:1); MS (FAB^+) *m/z*: 387.1 [100, $(M+H)^+$]; Acc. MS (FAB^+): 387.2509, $C_{24}H_{35}O_4$ requires 387.2491; ^1H NMR (400 MHz; $CDCl_3$); δ_H: 0.88 (s, 3H, CH_3), 1.19 (t, 3H, $CH_2C\underline{H}_3$, J = 7.8 Hz) 1.24-2.39 (m, 13H), 2.62 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.81-2.86 (m, 2H, CH_2), 3.48 (s, 3H, OCH_3), 3.85-3.98 (m, 4H, OCH_2CH_2O), 5.17 (s, 1H, OCH_2O), 6.78 (s, 1H, Ar-H) and 7.09 (s, 1H, Ar-H).

8.10.14 2-Ethylestrone (164)

To cold anhy. methanol (50 ml) under N_2, acetyl chloride (15.0 ml; 211 mmol) was syringed in drop-wise and when gas evolution ceased, **163** (9.5 g; 25 mmol) was added and cooled to 0°C. The mixture was sonicated for 15 min until the solution turned yellow. H_2O (50 ml) was added and the pale yellow precipitate formed was filtered. Solid $NaHCO_3$ was added to the filtrate and the organic layer was extracted with ethyl acetate (2×200 ml), washed twice with H_2O followed by brine dried and evaporated to give a pale yellow solid, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The white solid isolated was recrystallised from hot absolute ethanol to give **164** as white crystals (6.98 g, 95 %). R_f: 0.77 (ethyl acetate/hexane, 1:1); mp 182–183°C ([268]Lit mp 201-204°C); MS (FAB^+) *m/z*: 299.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 297.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 299.1994, $C_{20}H_{27}O_2$ requires 299.2011; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.22 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz) 1.25-2.54 (m, 13H), 2.59 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.80-2.85 (m, 2H, CH_2), 4.56 (s, 1H, OH), 6.52 (s, 1H, Ar-H) and 7.06 (s, 1H, Ar-H); ^{13}C NMR (100.5 MHz; $CDCl_3$) δ_C: 14.2 (CH_3), 22.3, 25.9, 26.6, 30.3, 30.5, 34.2 (all

CH_2), 38.7, 43.3 (both CH), 46.0 (C), 49.3 (CH), 56.4 (CH_3), 64.6, 65.2, 94.5 (all CH_2), 114.9, 119.2, 123.2 (all C), 125.2 (CH), 134.5, 146.3, 157.4 (all C) and 189.5 (CHO); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 281.7 nm; t_R = 9.0 min.

8.10.15 2-Ethyl-3-[(*N,N*-dimethylthiocarbamoyl)oxy]estra-1,3,5(10)-trien-17-one (165)

Using the procedure described for the preparation of compound **151**, NaH (740 mg; 18.5 mmol) and *N,N*-dimethylthiocarbomyl chloride (2.86 g; 23.1 mmol) were added to a DMF (100 ml) solution of **164** (4.6 g; 15 mmol). The mixture was heated at 80°C for 1 h, cooled to R.T and poured into cold water (200ml). The crude cream precipitate was filtered, dried under vacuum (6.66 g, 106% crude). An analytical sample was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the white solid isolated was recrystallised from ethyl acetate to give **165** as white needles. R_f: 0.67 (ethyl acetate/hexane, 1:1); mp 235–237°C; MS (FAB^+) *m/z*: 386.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 386.2146, $C_{23}H_{32}NO_2S$ requires 386.2154; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.19 (t, 3H, $CH_2\underline{CH_3}$, J = 7.8 Hz), 1.42-2.54 (m, 15H), 2.89 (q, 2H, $C\underline{H_2}CH_3$, J = 7.4 Hz), 3.34 (s, 3H, NCH_3), 3.47 (s, 3H, NCH_3), 6.73 (s, 1H, Ar-H) and 7.18 (s, 1H, Ar-H); Found C, 71.70; H, 8.08; N, 3.54; $C_{23}H_{31}NO_2S$ requires C, 71.65; H, 8.10; N, 3.63%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 249.8 nm; t_R = 3.2 min.

8.10.16 2-Ethyl-3-[(*N,N*-dimethylcarbamoyl)thio]estra-1,3,5(10)-trien-17-one (166)

Using the procedure described for the preparation of **152**, a suspension of **165** (5.0 g; 13 mmol) in mineral oil (200 ml) was heated under N_2 at 250-260°C overnight. The brown suspension formed was extracted with acetone (3×200 ml) and the crude brown solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The pale brown solid isolated was recrystallised from hot isopropyl alcohol to give **166**

as pale brown crystals (4.62 g; 92%). R_f: 0.56 (ethyl acetate/hexane, 1:1); mp 149-151°C; MS (FAB^+) *m/z*: 386.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 386.2147, $C_{23}H_{32}NO_2S$ requires 386.2154; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.18 (t, 3H, $CH_2C\underline{H}_3$, J = 7.8 Hz), 1.22-2.63 (m, 13H), 2.73 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.86-2.89 (m, 2H, CH_2), 3.01 (s, 3H, NCH_3), 3.11 (s, 3H, NCH_3), 6.79 (s, 1H, Ar-H) and 7.13 (s, 1H, Ar-H); Found C, 71.30; H, 8.05; N, 3.59; $C_{23}H_{31}NO_2S$ requires C, 71.65; H, 8.10; N, 3.63%; HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 275.7 nm; t_R = 8.4 min.

8.10.17 2-Ethyl-3-mercaptoestra-1,3,5(10)-trien-17-one (167)

Using the procedure described for the preparation of **153**, a solution of **166** (4.5 g; 12 mmol) in ethanol (300 ml) was hydrolysed with aqueous NaOH (13.1 g; 327 mmol). The precipitate formed was filtered, washed with water and dried to obtain a pale brown solid, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The pale brown solid isolated was recrystallised from isopropanol to give **167** as off white crystals (3.26 mg; 89%). R_f: 0.78 (ethyl acetate/hexane, 1:1); mp 119–121°C; MS (FAB^+) *m/z*: 314.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 313.0 [100, $(M-H)^+$]; Acc. MS (FAB^+): 314.1711, $C_{20}H_{26}OS$ requires 314.1704; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.23 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.36-2.54 (m, 13H), 2.65 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.82-2.85 (m, 2H, CH_2), 3.21 (s, 1H, SH), 7.04 (s, 1H, Ar-H) and 7.09 (s, 1H, Ar-H); Found C, 76.60; H, 8.22; $C_{20}H_{26}OS$ requires C, 76.38; H, 8.33%; HPLC: MeOH:H_2O (85:15); Flow rate = 2 ml/min, λ_{max} = 213.4, 242.7, 285.2 and 374.2 nm; t_R = 17.3 min.

8.10.18 2-Ethylestra-1,3,5(10)-trien-17-one-3-*S*-sulphamate (168)

Upon sulphamoylation by method 8.2.5 A, a DMF solution of **167** (100 mg; 0.32 mmol) gave a pale yellow solid, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the pale yellow solid isolated was recrystallised from ethyl acetate/hexane to get a pale yellow crystals of **168** (18 mg; 14%). R_f: 0.74

(ethyl acetate/hexane, 1:1); mp 136–137°C; νmax (KBr) cm^{-1}: 3400, 3300 (NH_2), 1610 (C=O), 1380 (SO_2); MS (e.s. in MeOH) *m/z*: 394.4 [10, $(M+H)^+$], 315.2 [100, $(M+H-HNSO_2)^+$]; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.22 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.26-2.54 (m, 13H), 2.81 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.83-2.84 (m, 2H, CH_2), 4.81 (s, 2H, NH_2), 7.11 (s, 1H, Ar-H) and 7.29 (s, 1H, Ar-H); HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 276.9, 322.0 and 368.2 nm; t_R = 3.2 min.

8.10.19 3-Benzylthio-2-ethylestra-1,3,5(10)-trien-17-one (169)

Using the procedure described for the preparation of **155**, a DMF (50 ml) solution of **167** (2.0 g; 6.4 mmol) was reacted with benzyl bromide (0.9 ml; 7.6 mmol) and the pale brown solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The white syrup obtained was further purified by preparative TLC to give **169** as white sticky foam (1.67 g, 65%). R_f: 0.77 (ethyl acetate/hexane, 1:1); mp 32–33°C; MS (FAB^+) *m/z*: 405.3 [100, $(M+H)^+$], 312.2 [20, $(C_{20}H_{25}OS+H)^+$], 91.0 [90, $(PhCH_2)^+$]; Acc. MS (FAB^+): 405.2236, $C_{27}H_{33}OS$ requires 405.2252; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.17 (t, 3H, $CH_2C\underline{H}_3$, J = 7.8 Hz), 1.21-2.54 (m, 13H), 2.69 (q, 2H, $C\underline{H}_2CH_3$, J = 7.8 Hz), 2.82-2.84 (m, 2H, CH_2), 4.06 (s, 2H, CH_2Ph), 7.04 (s, 1H, Ar-H), 7.11 (s, 1H, Ar-H) and 7.23-7.30 (m, 5H, Ph-H); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 233.1 and 377.9 nm; t_R = 4.7 min.

8.10.20 2-Ethylestra-1,3,5(10)-trien-17-one-3-sulphonylchloride (170)

Using the procedure described for the preparation of **156**, Cl_2 gas was bubbled into a cool suspension of **169** (1.0 g; 2.5 mmol) in glacial acetic acid (30 ml) and water (1 ml). The pale yellow syrup was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **170** as a pale yellow sticky foam, which solidified on standing (517 mg, 55%). R_f: 0.71 (ethyl acetate/hexane, 1:1); mp 94-96°C; MS (FAB^+) *m/z*: 381 [100, $(M(^{35}Cl)+H)^+$]; Acc. MS *m/z* (FAB^+): 381.1298, $C_{20}H_{26}{}^{35}ClO_3S$ requires 381.1291 and 383.1253, $C_{20}H_{26}{}^{37}ClO_3S$ requires 383.1283; ^1H

NMR (400 MHz; $CDCl_3$) δ_H: 0.93 (s, 3H, CH_3), 1.33 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.37-2.83 (m, 13H), 2.92 (q, 2H, $C\underline{H}_2CH_3$, J = 7.8 Hz), 3.11-3.15 (m, 2H, CH_2), 7.37 (s, 1H, Ar-H) and 7.58 (s, 1H, Ar-H).

8.10.21 2-Ethylestra-1,3,5(10)-trien-17-one-3-sulphonamide (171)

Using the procedure described for the preparation of **158**, cold aq. NH_4OH (5 ml) was added to a solution of **170** (120 mg; 0.32 mmol) in cold acetone (5 ml). The crude off-white solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The white foam isolated was further purified by preparative TLC to give **171** as fine white crystals (69 mg; 61%). R_f: 0.73 (ethyl acetate/hexane, 1:1); mp 132–133°C; MS (FAB^+) *m/z*: 362.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 360.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 362.1799, $C_{20}H_{28}NO_3S$ requires 362.1789; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.31 (t, 3H, $CH_2C\underline{H}_3$, J = 7.8 Hz), 1.33-2.63 (m, 13H), 2.92-2.94 (m, 2H, $C\underline{H}_2CH_3$), 2.99-3.01 (m, 2H, CH_2), 4.71 (s, 2H, NH_2), 7.30 (s, 1H, Ar-H) and 7.75 (s, 1H, Ar-H); Found C, 65.90; H, 7.33; N, 3.37; $C_{20}H_{27}NO_3S$ requires C, 66.45; H, 7.53; N, 3.87%;

8.10.22 2-Methoxy-1-[(*N,N*-dimethylthiocarbamoyl)oxy]benzene (172)

Using the procedure described for the preparation of **151**, NaH (1.93 g; 48.3 mmol) and dimethyl thiocarbomyl chloride (7.47 g; 60.4 mmol) was added to a DMF solution of 2-methoxyphenol (5.0 g; 11 mmol). The crude brown suspension was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **172** (7.33 mg; 86%) as a yellow syrup, which solidified on standing. R_f: 0.62 (ethyl acetate/hexane, 1:1); mp 62–63°C ([396]Lit. mp 61-62°C); MS (FAB^+) *m/z*: 212.0 [100, $(M+H)^+$], 180.0 [10, $(M-OCH_3)^+$]; Acc. MS (FAB^+): 212.0744, $C_{10}H_{14}NO_2S$ requires 212.0745; ^1H NMR (400 MHz; $CDCl_3$); δ_H : 3.35 (s, 3H, NCH_3), 3.45 (s, 3H, NCH_3), 3.82 (s, 3H, OCH_3) and 6.95-7.26 (m, 4H, Ar-H); Found C, 56.70; H, 6.21; N, 6.59; $C_{10}H_{13}NO_2S$ requires C, 56.85; H, 6.20; N, 6.63%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 249.8 nm; t_R = 2.6 min.

8.10.22 2-Methoxy-1-[(*N,N*-dimethylcarbamoyl)thio]benzene (173)

Using the procedure described for the preparation of **152**, a suspension of **172** (6.0 g; 28 mmol) in mineral oil (200 ml) was heated under N_2 at 280°C overnight. The crude yellow solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the pale yellow solid isolated was recrystallised from hot isopropyl alcohol to give **173** as off-white crystals (2.88 mg; 48%). R_f: 0.87 (ethyl acetate/hexane, 1:1); mp 92-93°C ([440]Lit. mp 89-91°C); MS (FAB^+) *m/z*: 212.0 [100, $(M+H)^+$]; Acc. MS (FAB^+): 212.0747, $C_{10}H_{14}NO_2S$ requires 212.0745; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 3.01 (s, 3H, NCH_3), 3.14 (s, 3H, NCH_3), 3.88 (s, 3H, OCH_3), 6.95-6.99 (m, 2H, Ar-H), 7.38-7.42 (m, 1H, Ar-H) and 7.46 (dd, 1H, Ar-H, J = 1.6, 7.4 Hz); Found C, 56.90; H, 6.22; N, 6.58; $C_{10}H_{13}NO_2S$ requires C, 56.85; H, 6.20; N, 6.63%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 281.7 nm; t_R = 2.7 min.

8.10.24 2-Methoxythiophenol (174)

Using the procedure described for the preparation of **153**, a solution of **173** (2.7 g; 13 mmol) in absolute ethanol (100 ml) was hydrolysed with NaOH (14.3 g; 358 mmol) in H_2O (20 ml). The crude brown syrup was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **174** as pale brown oil (1.32 g; 74%). R_f: 0.75 (hexane/ethyl acetate, 1:1); ([441]Lit. bp_{20} 115-116°C); MS (FAB^+) *m/z*: 279.0 [100, $(2M+H)^+$], 141.2 [60, $(M+H)^+$]; MS (FAB^-) *m/z*: 277.0 [100, $(2M-H)^-$], 139.1 [75, $(M-H)^-$]; Acc. MS (FAB^+): 141.1216, C_7H_8OS requires 141.1292; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 3.81 (s, 3H, SH exchanged with D_2O), 3.89 (s, 3H, OCH_3), 6.83-6.89 (m, 2H, Ar-H), 7.09-7.18 (m, 1H, Ar-H) and 7.24 (dd, 1H, Ar-H, J = 1.9 Hz and 7.8 Hz).

8.10.25 1-Benzylthio-2-methoxybenzene (175)

Using the procedure described for the preparation of **155**, to a DMF solution of **274** (1.5 g; 11 mmol), NaH (513 mg; 12.8 mmol) followed by benzyl bromide (1.53 ml; 12.8 mmol) was added. The white precipitate formed was filtered, washed with water, dried and purified by recrystallisation from ethyl acetate/hexane to give **175** as white

needles (2.16 g, 88%). R_f: 0.60 (ethyl acetate/hexane, 1:1); mp 69-70°C; MS (FAB^+) *m/z*: 230.0 [100, $(M+H)^+$], 91.1 [60, $(PhCH_2)^+$]; Acc. MS (FAB^+): 230.0770, $C_{14}H_{14}OS$ requires 230.0765; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 3.89 (s, 3H, OCH_3), 4.09 (s, 2H, CH_2Ph), 6.84-6.88 (m, 2H, Ar-H) and 7.17-7.31 (m, 2H, Ar-H). Found C, 72.90; H, 6.05; $C_{14}H_{14}OS$ requires C, 73.01; H, 6.13%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 285.2 nm; t_R = 9.9 min.

8.10.26 2-Methoxybenzenesulphonyl chloride (176)

Using the procedure described for the preparation of **156**, Cl_2 gas was bubbled in to a cool suspension of **175** (500 mg; 2.17 mmol) in acetic acid (50 ml) and water (8 ml). The crude pale yellow solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the pale yellow solid isolated was recrystallised from isopropyl alcohol to give **176** as fine white crystals (420 mg, 94%). R_f: 0.64 (ethyl acetate/hexane, 1:1); mp 58-59°C ([442]Lit. mp 56°C); MS (FAB^+) *m/z*: 206.2 [100, $(M(^{37}Cl)+H)^+$]; MS (FAB^-) *m/z*: 204.2 [100, $(M-H)^-$]; Acc. MS *m/z* (FAB^+): 204.2194, $C_7H_7{}^{35}ClO_3S$ requires 204.2201 and 206.2186, $C_7H_7{}^{37}ClO_3S$ requires 206.2191; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 3.75 (s, 3H, OCH_3), 6.84-6.88 (m, 1H, Ar-H), 6.96-7.29 (m, 1H, Ar-H), 7.32-7.35 (m, 1H, Ar-H) and 7.67 (dd, 1H, Ar-H, J = 1.9, 7.8 Hz).

8.10.27 2-Methoxybenzenesulphonamide (177)

Using the procedure described for the preparation of **158**, cold aq. NH_4OH (5 ml) was added to a solution of **176** (200 mg; 0.98 mmol) in cold acetone (5 ml). The crude yellow solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the white solid isolated was recrystallised from ethyl acetate/hexane to give **177** as fine white crystals (144 mg; 80%). R_f: 0.26 (ethyl acetate/hexane, 1:1); mp 137–138°C ([443]Lit. mp 171°C); MS (FAB^+) *m/z*: 188.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 186.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 188.0379, $C_7H_{10}NO_3S$ requires 188.0381; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 4.03 (s, 3H, OCH_3), 5.03 (s, 2H, NH_2), 6.99 (d, 1H, Ar-H, J = 7.0 Hz), 7.05-7.10 (m, 1H, Ar-H), 7.49-7.58 (m, 1H, Ar-H) and 7.92 (dd, 1H,

Ar-H, J = 1.6, 7.8 Hz); Found C, 44.30; H, 4.31; N, 6.90; $C_7H_9NO_3S$ requires C, 44.91; H, 4.85; N, 7.48%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 222.7 and 280.5 nm; t_R = 1.7 min.

8.10.28 17,17-Ethylenedioxy-2-formyl-3-(methoxymethoxy)-1,3,5[10]-estratriene (178)

A solution of **162** (17 g, 48 mmol) in freshly distilled THF (300 ml) was cooled to –78°C in a dry ice/acetone bath for 1 h and then treated with ^{sec}butyl lithium (12.0 ml, 142 mmol) dropwise over 4 h. After a further 4 h at this temperature DMF, freshly distilled from CaH_2 (15.0 ml, 190 mmol) was added and the mixture was allowed to come to R.T. overnight. Saturated aq. NH_4Cl solution (200 ml) was added followed by diethyl ether (400 ml). The extracts were washed with water (250 ml), brine (2×350 ml), dried and evaporated to give a white suspension. This was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give an off-white solid, which was recrystallised with ethanol to give **178** as white needles (17.89 g, 98 %). R_f: 0.73 (ethyl acetate/hexane, 1:1, a fluorescent spot); [¶]mp 104–105°C; MS (FAB$^+$) *m/z*: 387.1 [100, $(M+H)^+$]; Acc. MS (FAB$^+$): 387.2161, $C_{23}H_{31}O_5$ requires 387.2171; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (s, 3H, CH_3), 1.30-2.12 (m, 11H), 2.16-2.30 (m, 1H, CH), 2.35-2.46 (m, 1H, CH), 2.87-2.91 (m, 2H, CH_2), 3.51 (s, 3H, OCH_3), 3.87-3.98 (m, 4H, OCH_2CH_2O), 5.26 (s, 2H, OCH_2O), 6.91 (s, 1H, Ar-H), 7.77 (s, 1H, Ar-H) and 10.42 (s, 1H, CHO); ^{13}C NMR (100.5 MHz; $CDCl_3$) δ_C: 14.2 (CH_3), 22.3, 25.9, 26.6, 30.3, 30.5, 34.2 (all CH_2), 38.7, 43.3 (both CH), 46.0 (C), 49.3 (CH), 56.4 (CH_3), 64.6, 65.2, 94.5 (all CH_2), 114.9, 119.2, 123.2 (all C), 125.2 (CH), 134.5, 146.3, 157.4 (all C) and 189.5 (CHO), Found C, 70.80; H, 7.76; $C_{23}H_{30}O_5$ requires C, 71.48; H, 7.82%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 223.9, 268.7, 325.6 and 391 nm; t_R = 8.2 min.

[¶] Previously compound **178** was obtained as a thick clear syrup instead of a crystalline solid.[388]

8.10.29 17,17-Ethylenedioxy-2-hydroxy-3-(methoxymethyloxy)-1,3,5[10]-estratriene (179)

Method 1 :

The impurities present in *m*CPBA used in this reaction were removed by extraction with CH_2Cl_2 and brine. The purified *m*CPBA was dried at <50°C to remove water. A solution of *m*CPBA (14.7 g, 5.45 mmol, purified *ca* 95 % purity) in anhy. CH_2Cl_2 (300 ml) was added dropwise to a solution of **178** (16.5 g, 42.7 mmol) in CH_2Cl_2 (150 ml) containing Na_2HPO_4 (24.2 g, 171 mmol). The mixture was stirred for 3 h before pouring onto ice-water (300 ml), the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3×75 ml). The combined extracts were washed with saturated $NaHCO_3$ solution (100 ml), brine (2×100 ml) and evaporated. The resultant foam was taken up in degassed methanol (100 ml), and treated with NaOH (18 ml of 1M aq. solution). After 2 h of stirring, the mixture was neutralised by addition of aq. HCl (1M), methanol was removed by evaporation, ethyl acetate and water were added, the combined extracts were dried and evaporated to give a yellow syrup. This was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **179** as a clear syrup, which solidified on standing (12.8 g, 80 %). R_f: 0.53 (ethyl acetate/hexane, 1:1); mp 84–86°C ([388]Lit. mp 88°C); MS (FAB^+) *m/z*: 375.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 373.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 375.2096, $C_{22}H_{31}O_5$ requires 375.2093; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.87 (s, 3H, CH_3), 1.24-2.18 (m, 11H), 2.21-2.29 (m, 2H), 2.75-2.80 (m, 2H, CH_2), 3.51 (s, 3H, OCH_3), 3.87-3.99 (m, 4H, OCH_2CH_2O), 5.16 (s, 2H, OCH_2O), 5.74 (s, 1H, OH), 6.79 (s, 1H, Ar-H) and 6.90 (s, 1H, Ar-H); ^{13}C NMR (100.5 MHz; $CDCl_3$) δ_C: 14.3 (CH_3), 22.3, 26.1, 27.1, 29.0, 30.7, 34.2 (all CH_2), 38.8, 43.8 (both CH), 46.1 (C), 49.3 (CH), 56.3 (CH_3), 64.6, 65.2, 96.0 (all CH_2), 112.3, 115.8 (both CH_2), 119.4, 128.4, 135.2, 142.3, 144.1 (all C) and 145.1 (CHO); Found C, 69.90; H, 8.07; $C_{22}H_{30}O_5$ requires C, 70.56; H, 8.07%; HPLC: MeOH:H_2O (50:50); Flow rate = 2 ml/min, λ_{max} = 276.9 nm; t_R = 1.5 min.

Method 2[444]

8.10.30 2-Hydroxy-3-(methoxymethyloxy)-1,3,5[10]-estratriene (180)

*m*CPBA (970 mg, 5.62 mmol, purified *ca* 95 % purity) was added to a solution of **178** (1.0 g, 2.6 mmol) in a 1:3 mixture of $CHCl_3$: CH_2Cl_2 (27 ml) and the mixture was stirred at R.T. for 24 h. The resulting milky white mixture was worked up by consecutive extractions with CH_2Cl_2 (3×75 ml) and the combined organics were washed with saturated aq. Na_2SO_3 (100 ml) and aq. $NaHCO_3$ solutions (100 ml), brine (2×100 ml) and evaporated to give a yellow syrup, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **180** as an off-white syrup (520 mg, 61 %). R_f: 0.68 (ethyl acetate/hexane, 1:1); MS (FAB^+) *m/z*: 331.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 329.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 331.1842, $C_{20}H_{27}O_4$ requires 331.1856; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.87 (s, 3H, CH_3), 1.22-2.10 (m, 11H), 2.18-2.32 (m, 2H), 2.76-2.89 (m, 2H, CH_2), 3.51 (s, 3H, OCH_3), 5.14 (s, 2H, OCH_2O), 4.79 (s, 1H, OH), 6.93 (s, 1H, Ar-H) and 7.01 (s, 1H, Ar-H).

8.10.31 17,17-Ethylenedioxy-2-methoxy-3-(methoxymethyloxy)-1,3,5[10]-estratriene (181)

A stirred solution of **179** (12 .0g, 32.1 mmol) in anhy. DMF (200 ml) was treated with anhy. K_2CO_3 (44.34 g, 320.1 mmol) and, after a further 10 min, methyl iodide (22 ml, 153 mmol) followed by Bu_4NI (300 mg). After 20 h the mixture was poured onto brine (200 ml) and extracted with ethyl acetate (3×200 ml), washed with brine (5×100 ml), dried and evaporated to give a yellow oil, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The white solid isolated was recrystallised with ethyl acetate/hexane to give **181** as a white solid (11.3 g, 90 %). R_f: 0.70 (ethyl acetate/hexane, 1:1); mp 87–89°C; MS (FAB^+) *m/z*: 388.3 [100, $(M+H)^+$]; Acc. MS (FAB^+): 388.2241, $C_{23}H_{33}O_5$ requires 388.2249; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.89 (s, 3H, CH_3), 1.26-2.32 (m, 13H), 2.77-2.81 (m, 2H, CH_2), 3.52 (s, 3H, OCH_3), 3.86 (s, 3H, CH_3OAr), 3.88-3.99 (m, 4H, OCH_2CH_2O), 5.20 (s, 2H, OCH_2O), 6.85 (s, 1H, Ar-H) and 6.87 (s, 1H, Ar-H).

8.10.32 2-Methoxy-1,3,5[10]-estratriene-3-ol (182)

Using the procedure described for the preparation of **164**, a methanolic solution of **181** (11.0 g; 28.3 mmol) was deprotected by acetyl chloride (16.0 ml; 227 mmol). The crude pale yellow solid was recrystallised from ethanol to give **182** as white crystals (5.97 g, 70 %). R_f: 0.61 (ethyl acetate/hexane, 1:1); mp 184–186°C ([445]Lit. mp 188-191°C); MS (FAB^+) *m/z*: 301.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 299.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 301.1725, $C_{19}H_{25}O_3$ requires 301.1721; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, C18-CH_3), 1.35-2.55 (m, 13H), 2.80-2.84 (m, 2H, CH_2), 3.86 (s, 3H, OCH_3), 5.45 (s, 1H, OH), 6.66 (s, 1H, Ar-H) and 6.79 (s, 1H, Ar-H); ^{13}C NMR (100.5 MHz; $CDCl_3$) δC: 14.3 (CH_3), 22.0, 26.7, 27.0, 29.3, 32.0, 36.3 (all CH_2), 38.7 (CH), 44.6 (CH), 48.4 (C), 50.8 (CH), 56.3 (CH_3), 108.3, 114.9 (both CH), 129.5, 131.3, 143.8, 144.8 (all C) and 221.0 (CO); Found C, 75.60; H, 8.01; $C_{19}H_{24}O_3$ requires C, 75.97; H, 8.05%; HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 287.6 nm; t_R = 4.8 min.

8.10.33 2-Methoxy-3-[(*N,N*-dimethylthiocarbamoyl)oxy]estra-1,3,5(10)-trien-17-one (183)

Using the procedure described for the preparation of **151**, NaH (479 mg; 11.9 mmol) and *N,N*-dimethylthiocarbamyl chloride (1.85 g; 15.0 mmol) were added to a DMF solution of **182** (3.0 g; 10 mmol). The crude pale yellow precipitate was filtered and dried to get a pale yellow solid, which was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 1:1 gradient). The white solid isolated was recrystallised from isopropyl alcohol to give **183** as white needles (2.85 g, 74%). R_f: 0.39 (ethyl acetate/hexane, 1:2); mp 217–219°C; MS (FAB^+) *m/z*: 388.1 [100, $(M+H)^+$], 87.9 [50, $((CH_3)_2NC=S+H)^+$]; Acc. MS (FAB^+): 388.1938, $C_{22}H_{30}NO_3S$ requires 388.1946; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.26-2.55 (m, 13H), 2.84-2.86 (m, 2H, CH_2), 3.34 (s, 3H, NCH_3), 3.46 (s, 3H, NCH_3), 3.80 (s, 3H, OCH_3), 6.76 (s, 1H, Ar-H) and 6.89 (s, 1H, Ar-H); HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 249.8 and 281.7 nm; t_R = 4.0 min.

8.10.34 2-Methoxy-3-[(*N,N*-dimethylcarbamoyl)thio]estra-1,3,5(10)-trien-17-one (184)

Using the procedure described for the preparation of **152**, a suspension of **183** (2.7 g; 7.0 mmol) in mineral oil (200 ml) was heated under N_2 at 270°C overnight. The crude brown solid was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the pale brown solid isolated was recrystallised from ethyl acetate to give **184** as off-white crystals (1.21 g; 45%). R_f: 0.24 (ethyl acetate/hexane, 3:4); mp 209-211°C; MS (FAB^+) *m/z*: 388.1 [100, $(M+H)^+$]; Acc. MS (FAB^+): 388.1946, $C_{22}H_{30}NO_3S$ requires 388.1946; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.16-2.55 (m, 13H), 2.84-2.86 (m, 2H, CH_2), 3.01 (s, 3H, NCH_3), 3.13 (s, 3H, NCH_3), 3.85 (s, 3H, OCH_3), 6.89 (s, 1H, Ar-H) and 7.19 (s, 1H, Ar-H); HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 291.1 nm; t_R = 4.5 min.

8.10.35 3-Mercapto-2-methoxyestra-1,3,5(10)-trien-17-one (185)

Using the procedure described for the preparation of **153**, a solution of **184** (1.0 g; 2.6 mmol) in ethanol (300 ml) was hydrolysed by aqueous NaOH (2.9 g; 72 mmol) in H_2O (30 ml). The precipitate was filtered, washed with water and dried to get a brown solid, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The pale brown solid isolated was recrystallised from hot ethyl acetate to give **185** as off-white crystals (201 mg; 25%). R_f: 0.77 (ethyl acetate/hexane, 1:1); mp 157-159°C; MS (FAB^+) *m/z*: 316.1 [100, $(M)^+$]; MS (FAB^-) *m/z*: 315.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 316.1495, $C_{19}H_{25}O_2S$ requires 316.1497; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.21-2.55 (m, 13H), 2.70-2.82 (m, 2H, CH_2), 3.72 (s, 1H, SH), 3.86 (s, 3H, OCH_3), 6.78 (m, 1H, Ar-H) and 6.99 (s, 1H, Ar-H); Found C, 71.50; H, 7.61; $C_{19}H_{24}O_2S$ requires C, 72.11; H, 7.64%; HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 213.4, 243.9 and 361.8 nm; t_R = 7.9 min.

8.10.36 2-Methoxyestra-1,3,5(10)-trien-17-one-3-*S*-sulphamate (186)

Upon sulphamoylation by method 8.2.5 A, a DMF solution of **185** (50 mg; 0.16 mmol) gave a pale brown solid, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient). The pale yellow solid isolated was recrystallised from ethyl acetate/hexane to get a pale yellow crystals of **186** (9 mg; 14%). R_f: 0.69 (ethyl acetate/hexane, 1:1); mp 118-119°C; MS (e.s. in MeOH) *m/z*: 396.4 [20, $(M+H)^+$], 317.1 [45, $(M+H-HNSO_2)^+$]; NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.20-2.54 (m, 13H), 2.72-2.85 (m, 2H, CH_2), 3.89 (s, 3H, OCH_3), 5.22 (s, 2H, NH_2), 6.95 (s, 1H, Ar-H) and 6.99 (s, 1H, Ar-H); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 209.8 nm; t_R = 2.4 min.

8.10.37 3-Benzylthio-2-methoxyestra-1,3,5(10)-trien-17-one (187)

Using the procedure described for the preparation of **155**, a DMF (10 ml) solution of **185** (100 mg; 0.38 mmol) was stirred with benzyl bromide (0.5 ml; 0.38 mmol) at R.T. for 3 h under N_2. The crude brown syrup was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) and the pale brown sticky foam isolated was further purified by preparative TLC to get **187** as a pale yellow solid (27 mg, 21%). R_f: 0.59 (ethyl acetate/hexane, 1:1); mp 69-71°C; MS (FAB^+) *m/z*: 406.3 [50, $(M+H)^+$], 91.1 [100, $(PhCH_2)^+$]; Acc. MS (FAB^+): 406.1951, $C_{26}H_{30}O_2S$ requires 460.1966; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.24-2.59 (m, 13H), 2.76-2.84 (m, 2H, CH_2), 3.86 (s, 3H, OCH3), 4.07 (s, 2H, SCH_2), 6.78 (s, 1H, Ar-H), 6.97 (s, 1H, Ar-H) and 7.26-7.38 (m, 5H, Ph-H); HPLC: MeOH:H_2O (85:15); Flow rate = 2 ml/min, λ_{max} = 294.7 nm; t_R = 17.1 min.

8.10.38 2-Ethenyl-17,17-ethylenedioxy-3-*O*-methoxymethylene-1,3,5[10]-estratriene (188)

To a solution of methyltriphenylphosphonium iodide (3.92 g, 9.71 mmol) in freshly distilled THF (40 ml), potassium ^{tert}butoxide in THF (1.21 ml, 9.71 mmol) was added and stirred at R.T. for 1 h. To the mixture, **178** (1.5 g, 3.9 mmol) dissolved in THF (10

ml) was added and left to stir for 2 h. The milky white mixture was quenched with H_2O and extracted with ethyl acetate. The combined organics were washed with water, dried and evaporated to give a yellow suspension, which was purified by flash chromatography (ethyl acetate/hexane, 5:1 to 1:1 gradient) to give **188** as colourless syrup (1.22 g; 82 %). R_f: 0.77 (ethyl acetate/hexane, 1:2); MS (FAB^+) *m/z*: 384.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 383.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 384.3367, $C_{24}H_{33}O_4$ requires 384.3391; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.88 (s, 3H, CH_3), 1.24-2.11 (m, 11H), 2.15-2.35 (m, 1H, CH), 2.36-2.45 (m, 1H, CH), 2.83-2.87 (m, 2H, CH_2), 3.48 (s, 3H, OCH_3), 3.88-3.99 (m, 4H, OCH_2CH_2O), 5.17 (s, 2H, OCH_2O), 5.22 (d, 1H, $C\underline{H}_2$=CH, J = 11 Hz), 5.71 (d, 1H, $C\underline{H}_2$=CH, J = 9.0 Hz), 6.81 (s, 1H, Ar-H), 7.01 (dd, 1H, CH_2=C$\underline{H}$, J = 9.0, 11 Hz) and 7.41 (s, 1H, Ar-H); HPLC: MeOH:H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 215.7, 254.5 and 304.2 nm; t_R = 9.0 min.

8.11 Derivatives of estrone-3-sulphamide

8.11.1 3-(2'-Phenyl-4'-quinazolinyloxy)estra-1,3,5(10)-trien-17-one (189)

A solution of estrone (3.0 g; 11 mmol) in dry diglyme (100 ml) was treated with NaH (60% dispersion in mineral oil, 591 mg; 14.8 mmol). The mixture was stirred under N_2 until the evolution of H_2 ceased. To this suspension was added 4-chloro-2-phenylquinazoline (3.2 g; 13.3 mmol) and stirred and boiled under reflux for 3 h. The cooled pale yellow mixture was poured into cold water (500 ml). The precipitate formed was then filtered, washed with water and dried. The crude yellow solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the pale yellow solid isolated was recrystallised from isopropanol to give **189** as pale yellow fine crystals (4.21 g; 80%). R_f: 0.52 (ethyl acetate/hexane, 1:1); mp 189-191°C ([194]Lit. mp 193-195°C); MS (FAB^+) *m/z*: 475.1 [100, $(M+H)^+$]; Acc. MS (FAB^+): 475.2386, $C_{32}H_{31}N_2O_2$ requires 475.2385; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.98 (s, 3H, CH_3), 1.25-2.58 (m, 13H), 2.98-3.06 (m, 2H, CH_2), 7.13 (d, 1H, C_4-H, J = 1.9 Hz), 7.20 (dd, 1H, C_2-H, J = 2.3, 8.6 Hz), 7.40-7.44 (m, 4H, C_1-H, meta and para H of 2'-phenyl ring), 7.60 (t, 1H, C6'-H, J = 8.2 Hz), 7.89 (t, 1H, C7'-H, J = 8.2 Hz), 8.35 (d,

1H, C8'-H, J = 8.2 Hz) and 8.37-8.39 (m, 3H, C5'-H, and ortho H of 2'-phenyl ring); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 238 and 286.4 nm; t_R = 3.2 min.

8.11.2 3-[4'-Oxo-2'-phenyl-3'(4*H*)-quinazolinyl]estra-1,3,5(10)-trien-17-one (190)

A suspension of **189** (4.0 g; 8.4 mmol) in heavy mineral oil (100 ml) was stirred and heated at 300–310°C for 7 h under N_2. The mixture was cooled to R.T, diluted with petroleum ether (40–60°C) and the precipitate formed was filtered and dried under vacuum. The crude yellow solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the pale yellow solid isolated was recrystallised from isopropyl alcohol to give **190** as fine pale yellow crystals (3.18 g; 79%).[194] R_f: 0.61 (ethyl acetate/hexane, 3:4); mp 251-253°C; MS (FAB^+) *m/z*: 475.2 [100, $(M+H)^+$]; Acc. MS (FAB^+): 475.2389, $C_{32}H_{31}N_2O_2$ requires 475.2385; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.89 (s, 3H, CH_3), 1.24-2.54 (m, 13H), 2.74-2.87 (m, 2H, CH_2), 6.84-6.87 (m, 2H), 7.19-7.27 (m, 2H), 7.35-7.37 (m, 2H), 7.51-7.55 (m, 1H), 7.79-7.82 (m, 2H) and 8.35 (d, 1H, J = 7.8 Hz); HPLC: MeOH:H_2O (80:20); Flow rate = 2 ml/min, λ_{max} = 226.3 and 276.9 nm; t_R = 4.8 min.

8.11.3 3-Aminoestra-1,3,5(10)-trien-17-one (191)

A solution of **190** (3.0 g; 6.3 mmol) dissolved in ethanol (150 ml) was treated with 40% aq. NaOH solution (25.0 ml; 177 mmol) and boiled under reflux for 7 h under N_2. This solution was cooled in ice, treated with 12M aq. HCl (50 ml) and allowed to stand overnight at R.T. The mixture was stirred and boiled under reflux for 1.5 h, cooled and the NaCl precipitate was filtered out, washed thoroughly and discarded. The filtrate and washings were concentrated under reduced pressure, poured into water and filtered. The filtrate was made alkaline with NaOH solution, saturated with K_2CO_3 and the precipitate formed was filtered out washed well with water and dried under vacuum. The crude pale brown solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the pale yellow solid isolated was recrystallised

from isopropanol to give **191** as brown flaky crystals (274 mg, 16%). R_f: 0.44 (ethyl acetate/hexane, 1:1); mp 193-195°C ([194]Lit. mp 183-190°Cl); MS (FAB^+) *m/z*: 270.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 268.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 270.1834, $C_{18}H_{24}NO$ requires 270.1858; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.90 (s, 3H, CH_3), 1.20-2.55 (m, 13H), 2.81-2.95 (m, 2H, CH_2), 3.54 (s, 2H, NH_2), 6.45 (d, 1H, C_4-H, J = 2.3 Hz), 6.52 (dd, 1H, C_2-H, J = 2.3, 8.2 Hz) and 7.08 (d, 1H, C_1-H, J = 8.2 Hz); HPLC: MeOH:H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 236.8 and 290 nm; t_R = 3.9 min.

8.11.4 Estra-1,3,5(10)-trien-17-one-3-sulphamide (192)

Upon sulphamoylation using method 8.2.5 A, compound **191** (100 mg; 0.37 mmol) gave a crude brown solid, which was purified by preparative TLC (ethyl acetate/hexane, 1:1) and the pale yellow solid isolated was recrystallised from ethyl acetate/hexane to give **192** as cream crystals (22 mg; 17%). R_f: 0.26 (ethyl acetate/hexane, 1:1); mp 214-216°C ([194]Lit. mp 215-220°C–dec.); MS (FAB^+) *m/z*: 349.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 347.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 349.1560, $C_{18}H_{25}N_2O_3S$ requires 349.1586; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.21-2.55 (m, 13H), 2.89-2.93 (m, 2H, CH_2), 4.67 (s, 2H, NH_2), 6.99 (s, 1H, NH), 7.26-7.28 (m, 2H, Ar-H) and 7.30-7.31 (m, 1H, Ar-H); Found HPLC: MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 232.1 and 278.1 nm; t_R = 2.5 min.

8.11.5 2-Ethyl-3-(2'-phenyl-4'-quinazolinyloxy)estra-1,3,5(10)-trien-17-one (193)

As previously described for the preparation of compound **189**, a solution of **164** (2.1 g; 7.0 mmol) in dry diglyme (80 ml) was treated with NaH (375 mg; 9.37 mmol) and 4-chloro-2-phenylquinazoline (2.0 g; 8.5 mmol). The precipitate formed was filtered, washed with water and dried under vacuum. The crude white solid was purified by recrystallisation from isopropyl alcohol to give **193** as white fine crystals (2.49 g; 70%). R_f: 0.78 (ethyl acetate/hexane, 1:1); mp 147-149°C; MS (FAB^+) *m/z*: 503.0 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 501.0 [100, $(M-H)^-$]; Acc. MS (FAB^+): 503.2712,

$C_{34}H_{35}N_2O_2$ requires 503.2708; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.98 (s, 3H, CH_3), 1.78 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.22-2.53 (m, 13H), 2.59 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.93-2.96 (m, 2H, CH_2), 7.07 (s, 1H, Ar-H), 7.29 (s, 1H, Ar-H), 7.32-7.43 (m, 3H, meta and para H of 2'-phenyl ring), 7.61 (t, 1H, C6'-H, J = 8.2 Hz), 7.89 (t, 1H, C7'-H, J = 8.2 Hz), 8.05 (d, 1H, C8'-H, J = 8.6 Hz) and 8.34-8.38 (m, 3H, C5'-H, and ortho H of 2'-phenyl ring); Found C, 80.50; H, 6.67; N, 5.55; $C_{34}H_{34}N_2O_2$ requires C, 81.24; H, 6.82, N, 5.57; HPLC: MeOH/H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 209.8 nm; t_R = 18.5 min.

8.11.6 2-Ethyl-3-[4'-oxo-2'-phenyl-3'(4*H*)-quinazolinyl]estra-1,3,5(10)-trien-17-one (194)

As previously described for the preparation of compound **190**, a suspension of **193** (2.0 g; 4.0 mmol) in heavy mineral oil (80 ml) was heated at 320–330°C overnight under N_2. The pale brown precipitate formed was filtered out, washed with petroleum ether (40–60°C), dried and purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The white solid isolated was recrystallised from isopropyl alcohol to give **194** as white crystals (1.13 g, 57%). R_f: 0.56 (ethyl acetate/hexane, 1:1); mp 151-152°C; MS (FAB$^+$) *m/z*: 503.1 [100, $(M+H)^+$]; MS (FAB$^-$) *m/z*: 501.1 [100, $(M-H)^-$]; Acc. MS (FAB$^+$): 503.2617, $C_{34}H_{35}N_2O_2$ requires 503.2698; ^{1}H NMR (400 MHz; $CDCl_3$) δ_H: 0.90 (s, 3H, CH_3), 1.07 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.23-2.54 (m, 14H), 2.76 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.82-2.90 (m, 2H, CH_2), 6.84 (s, 1H, Ar-H), 7.12 (s, 1H, Ar-H), 7.20-7.28 (m, 2H), 7.31-7.37 (m, 2H), 7.50-7.57 (m, 1H), 7.79-7.84 (m, 2H) and 8.36 (d, 1H, J = 8.2 Hz); HPLC: MeOH/H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 276.9 nm; t_R = 3.6 min.

8.11.7 3-Amino-2-ethylestra-1,3,5(10)-trien-17-one (195)

A solution of **194** (1.0 g; 2.0 mmol) in absolute ethanol (50 ml) was hydrolysed with 40% aq. NaOH solution (14 ml) as previously described for the preparation of compound **191**. The precipitate formed was filtered out washed with water and dried

under vacuum. The crude pale brown solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the beige solid isolated was recrystallised from ethyl acetate/hexane to give **195** as pale brown fine crystals (212 mg, 36%). R_f: 0.74 (ethyl acetate/hexane, 1:1); mp 149-150°C; MS (FAB^+) *m/z*: 298.1 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 296.1 [100, $(M-H)^-$]; Acc. MS (FAB^+): 298.2097, $C_{20}H_{28}NO$ requires 298.2093; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.24 (t, 3H, $CH_2C\underline{H}_3$, J = 7.4 Hz), 1.25-2.43 (m, 15H reduced to 13H with D_2O), 2.51 (q, 2H, $C\underline{H}_2CH_3$, J = 7.8 Hz), 2.81-2.82 (m, 2H, CH_2), 6.46 (s, 1H, Ar-H) and 7.00 (s, 1H, Ar-H); HPLC: MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 291.1 nm; t_R = 7.6 min.

8.11.8 2-Ethylestra-1,3,5(10)-trien-17-one-3-sulphamide (196)

Upon sulphamoylation using method 8.2.5 A, compound **195** (200 mg; 0.67 mmol) gave a crude pale yellow solid, which was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient). The off-white solid isolated was recrystallised from THF/hexane to give **196** as off-white crystals (73 mg, 29%). R_f: 0.38 (ethyl acetate/hexane, 1:1); mp 153-154°C; νmax (KBr) cm^{-1}: 3392 (NH_2), 3243 (N-H), 1725 (C=O), 1252 (SO_2); MS (FAB^+) *m/z*: 376.2 [100, $(M+H)^+$]; 296.3 [35, $(C_{20}H_{27}NO+H)^+$]; MS (FAB^-) *m/z*: 375.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 376.1807, $C_{20}H_{28}N_2O_3S$ requires 376.1821; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, CH_3), 1.22 (t, 3H, $CH_2C\underline{H}_3$, J = 7.8 Hz), 1.25-2.55 (m, 13H), 2.63 (q, 2H, $C\underline{H}_2CH_3$, J = 7.4 Hz), 2.83-2.91 (m, 2H, CH_2), 4.71 (s, 2H, NH_2), 6.19 (s, 1H, NH), 7.16 (s, 1H, Ar-H) and 7.23 (s, 1H, Ar-H); Found C, 63.10; H, 7.46; N, 7.17; $C_{20}H_{28}N_2O_3S$ requires C, 63.80; H, 7.50, N, 7.44%; HPLC: MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 280.5 nm; t_R = 3.5 min.

8.11.9 2-Ethylestra-1,3,5(10)-trien-17-ol-3-sulphamide (197)

To a solution of **196** (25.0 mg; 0.07 mmol) in isopropanol (1.5 ml) and THF (0.5 ml) was added $NaBH_4$ (3.8 mg; 0.1 mmol) at 0°C. The mixture was stirred for 30 min at

0°C, quenched with aq. NH_4Cl (5 ml). H_2O (30 ml) was added and the precipitate formed was filtered out, washed with water and dried under vacuum. The crude white solid was purified by preparative TLC to give **197** as a white fine powder (9 mg; 36%). R_f: 0.40 (ethyl acetate/hexane, 1:1); mp 138-140°C; νmax (KBr) cm^{-1}: 3446 (OH), 3370 (NH_2), 3261 (N-H), 1260 (SO_2); MS (FAB^+) *m/z*: 379.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 377.3 [100, $(M-H)^-$]; Acc. MS (FAB^+): 379.2015, $C_{20}H_{31}N_2O_3S$ requires 379.2055; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.91 (s, 3H, C18-CH_3), 1.22 (t, 3H, CH_2CH_3, J = 7.8 Hz), 1.25-2.55 (m, 13H), 2.63 (q, 2H, CH_2CH_3, J = 7.4 Hz), 2.83-2.91 (m, 2H, CH_2), 3.73 (t, 1H, C17-H, J = 8.6 Hz), 4.63 (s, 2H, NH_2), 6.12 (s, 1H, NH), 7.16 (s, 1H, Ar-H) and 7.21 (s, 1H, Ar-H).

8.11.10 2-Methoxy-3-(2'-phenyl-4'-quinazolinyloxy)estra-1,3,5(10)-trien-17-one (198)

As previously described for the preparation of compound **189**, a solution of **182** (2.5 g; 8.3 mmol) in dried diglyme (70 ml) was treated with NaH (443 mg; 11.1 mmol) and 4-chloro-2-phenylquinazoline (2.41 g; 9.99 mmol). The precipitate formed was filtered, washed with water and dried under vacuum. The crude pale yellow solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the white solid isolated was recrystallised from isopropyl alcohol to give **198** as white crystals (3.49 g, 83%). R_f: 0.62 (ethyl acetate/hexane, 1:1); mp 191–192°C; MS (FAB^+) *m/z*: 505.1 [100, $(M+H)^+$]; Acc. MS (FAB^+): 505.2494, $C_{33}H_{33}N_2O_3$ requires 505.2491; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.99 (s, 3H, CH_3), 1.22-2.58 (m, 13H), 2.98-3.02 (m, 2H, CH_2), 3.71 (s, 3H, OCH_3), 7.01 (s, 1H, Ar-H), 7.03 (s, 1H, Ar-H), 7.39-7.41 (m, 3H, meta and para H of 2'-phenyl ring), 7.59 (t, 1H, C6'-H, J = 7.0 Hz), 7.88 (t, 1H, C7'-H, J = 7.0 Hz), 8.04 (d, 1H, C8'-H, J = 8.6 Hz), 8.31-8.33 (m, 2H, ortho H of 2'-phenyl ring) and 8.39 (d, 1H, C5'-H, J = 8.2 Hz); HPLC: MeOH/H_2O (90:10); Flow rate = 2 ml/min, λ_{max} = 255.7 and 286.4 nm; t_R = 8.6 min.

8.11.11 2-Methoxy-3-[4'-oxo-2'-phenyl-3'(4*H*)-quinazolinyl]estra-1,3,5(10)-trien-17-one (199)

As previously described for the preparation of compound **190**, a suspension of **198** (3.2 g; 6.4 mmol) in heavy mineral oil (100 ml) was heated at 310–320°C overnight under N_2. The precipitate formed was filtered, washed with petroleum ether (40–60°C) and dried under vacuum. The crude pale brown solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the yellow solid isolated was recrystallised from isopropyl alcohol to give **199** as pale yellow crystals (1.91 g, 60%). R_f: 0.43 (ethyl acetate/hexane, 1:1); mp 144-145°C; MS (FAB^+) *m/z*: 505.3 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 503.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 505.2472, $C_{33}H_{33}N_2O_3$ requires 505.2491; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.93 (s, 3H, CH_3), 1.26-2.59 (m, 14H), 2.81-2.87 (m, 2H, CH_2), 3.63 (s, 3H, OCH_3), 6.71 (s, 1H, Ar-H), 6.85 (s, 1H, Ar-H), 7.19-7.28 (m, 2H), 7.37-7.39 (m, 2H), 7.49-7.59 (m, 1H), 7.79-7.83 (m, 2H) and 8.35 (d, 1H, J = 7.4 Hz); HPLC: MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 238 and 287.6 nm; t_R = 3.3 min.

8.11.12 3-Amino-2-methoxyestra-1,3,5(10)-trien-17-one (200)

A solution of **199** (1.5 g; 3.0 mmol) in ethanol (90 ml) was hydrolysed with 40% aq. NaOH solution (21 ml) as previously described for the preparation of compound **191**. The precipitate formed was filtered, washed with water and dried under vacuum. The crude brown solid was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 2:1 gradient) and the brown solid isolated was recrystallised from isopropyl alcohol to give **200** as pale brown crystals (589 mg, 66%). R_f: 0.62 (ethyl acetate/hexane, 1:1); mp 129-130°C; MS (FAB^+) *m/z*: 299.2 [100, $(M+H)^+$]; Acc. MS (FAB^+): 299.1986, $C_{19}H_{26}NO_2$ requires 299.1966; ^1H NMR (400 MHz; DMSO-d_6) δ_H: 0.81 (s, 3H, CH_3), 1.24-2.48 (m, 13H), 2.58-2.69 (m, 2H, CH_2), 3.32 (s, 3H, OCH_3), 4.42 (s, 2H, NH_2), 6.30 (s, 1H, Ar-H) and 6.64 (s, 1H, Ar-H); HPLC: MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 292.3 and 348.2 nm; t_R = 4.9 min.

8.11.13 2-Methoxyestra-1,3,5(10)-trien-17-one-3-sulphamide (201)

Upon sulphamoylation using method 8.2.5 A, compound **200** (400 mg; 1.34 mmol) gave a crude brown solid, which was purified by flash chromatography (ethyl acetate/hexane, 6:1 to 1:1 gradient). The white solid isolated was recrystallised from ethyl acetate/hexane to give **201** as white crystals (72 mg; 14%). R_f: 0.28 (ethyl acetate/hexane, 1:1); mp 98-99°C; νmax (KBr) cm^{-1}: 3370 (NH_2), 3261 (N-H), 3940 (OCH_3), 1650 (C=O), 1252 (SO_2); MS (FAB^+) *m/z*: 379.2 [100, $(M+H)^+$]; MS (FAB^-) *m/z*: 377.2 [100, $(M-H)^-$]; Acc. MS (FAB^+): 379.1668, $C_{19}H_{27}N_2O_4S$ requires 379.1679; ^1H NMR (400 MHz; $CDCl_3$) δ_H: 0.92 (s, 3H, CH_3), 1.36-2.55 (m, 13H), 3.85 (s, 3H, OCH_3), 2.89-2.94 (m, 2H, CH_2), 4.61 (s, 2H, NH_2), 6.81 (s, 1H, NH), 6.83 (m, 1H, Ar-H) and 7.26 (s, 1H, Ar-H); HPLC: Mobile phase, MeOH/H_2O (70:30); Flow rate = 2 ml/min, λ_{max} = 287.6 nm; t_R = 2.8 min.

Summary

A steroid sulphatase inhibitor can be used as a new form of treatment for hormone dependent breast cancer (HDBC) by virtue of its ability to inhibit estrone sulphatase (E1-STS), which catalyses the hydrolysis of estrone sulphate to estrone, and dehydroepiandrostenedione sulphatase (DHA-STS), which has an important role in regulating the production of the estrogenic steroid androstenediol. The first potent steroid sulphatase inhibitor, estrone 3-*O*-sulphamate, (EMATE) is orally active and an irreversible active site-directed inhibitor with *in vivo* antitumour properties. However, EMATE proved to be highly estrogenic. Therefore, a number of strategies have been adopted to design and synthesise potent non-steroidal, non-estrogenic inhibitors and to explore further structure-activity relationship for sulphamate based steroid sulphatase inhibitors.

4-Methylcoumarin-7-*O*-sulphamate (COUMATE) is an orally active, non-steroidal, non-estrogenic and an active site-directed inhibitor, showing inhibitory activity against both E1-STS and DHA-STS in MCF-7 cells and placental microsomes. This led to the design of series of COUMATE analogues. A structure-activity study was carried out by synthesising coumarin analogues with longer alkyl chains and other functionalities at the C-4 and/or C-3 position of the coumarin ring. Another series of tricyclic coumarin sulphamates, which structurally mimic the ABCD rings of the steroid were also, synthesised as potentially more effective inhibitors than EMATE and COUMATE. Although much success and progress have been with coumarin sulphamates, other heterocyclic ring containing sulphamates are also of considerable interest as potential STS inhibitors. The indole ring system is useful to explore, since incorporation of an indole moiety in compounds intended for endocrine therapy has not been reported so far. Therefore several novel, 1/2 or 3-substituted indole sulphamates were syntheisesd.

The best analogues in the C-3 and C-4 alkyl series were found to be compounds with 7-9 and 6-9 carbon containing alkyl chains, respectively inhibiting the E1-STS activity in MCF-7 cells at 0.01 μM by 85-91% and >90%. (c.f. 35% for COUMATE). The 3-benzyl-4-methylcoumarin-7-*O*-sulphamate **(95)** was found to be a potent inhibitor with

an IC_{50} of 8 nM *in vivo*. Other compounds such as 4-methyl-3-phenethylcoumarin-7-*O*-sulphamate **(18)** 4-isopropylcoumarin-7-*O*-sulphamate **(32)** were also identified to be potent in this series with 91% and 86% inhibition respectively in placental microsomes at 0.1 μM concentration. In the tricyclic coumarin-based analogues the compounds synthesised possess a third ring that contain between 5 and 15 carbons (665-6615 COUMATES). These compounds were found to be more powerful non-steroidal, non-estrogenic and active site-directed inhibitors than EMATE, COUMATE and its analogues. The most potent compound in this series, 6610 COUMATE **(122)** has an IC_{50} of 1 nM *in vitro* followed by 669 COUMATE **(119)** with an IC_{50} of 2.4 nM. Interestingly, 6615 COUMATE **(131)** the least active compound with an IC_{50} of 370 nM *in vitro* proved to be the most active congener *in vivo*. This could be due to the difference in *in vitro* and *in vivo* biological testing. In an animal model the entry of this compound into tissues being facilitated by its higher lipophilicity and taken into cells more effectively. Therefore, its greater activity might be attributed to a combination of factors *in vivo*.

The higher potencies observed for the alkyl series of compounds can be attributed to their bicyclic system mimicking the A/B rings of steroid and that the 3- or 4- alkyl substituents interacting with the amino acid residues at the active site, which normally recognise the C/D rings of steroid. The greater inhibition observed for the tricyclic COUMATEs could be due to the fact that their third cycloalkenone ring folds in such a way that they mimic the C/D ring conformation of the steroid and hence have a better recognition for binding at the enzyme active site. It is promising that at least one of these tricyclic coumarin sulphamates seems likely to be useful whereby the efficacy of treating hormone dependent breast cancer with a steroid sulphatase inhibitor can be fully evaluated. Additionally, sulphatase inhibition may have potential therapeutic benefit in the immune system and for neuro-degenerative diseases, such as Alzheimer's.

In addition, The X-ray crystal structures of compounds **119** and **122** were determined to explore the conformation of their respective third rings at least in the crystalline

state. The lipophilicity of the tricyclic compounds was also studied by calculating the log P values by an HPLC technique. As expected, this study revealed that the lipophilicity of the tricyclic compounds increased steadily with the increase in the number of carbon atom in the third ring and sulphamoylation reduced the log P of the corresponding hydroxycoumarin. Interestingly, the log P value obtained for the most potent compound in this series *in vitro*, **122** was found to be 3.92, which turned out to be the same as that of EMATE (3.92).

In addition to inhibiting the sulphatase enzyme as a treatment for hormone dependent breast tumours, inhibiting the microtubules, which are involved in diverse cellular processes such as cell division, locomotion and intracellular transport is currently gaining attention as a potential target for hormone dependent and independent cancers in general. As a consequence any selective agent that can cause inhibition of tubulin polymerisation, angiogenesis and induction of apoptosis is a popular choice for new chemotherapeutics. 2-Methoxyestradiol (2-MeOE2), a natural estradiol metabolite, is non-estrogenic, known to induce G2/M phase cell cycle arrest and bcl-2 phosphorylation, two key markers of apoptosis. It is known that the synthetic sulphamate derivatives of this molecule 2-MeOEMATE, 2-MeOE2EMATE, 2-EtEMATE and 2-EtE2EMATE are highly potent, non-estrogenic steroid sulphatase inhibitors and as expected are shown to trigger apoptosis and inhibit the tubulin polymeraisation and angiogenesis. It has been recognised that the potent activities observed for these synthetic sulphamates compared to those of 2-MeOE2 are due to the presence of the sulphamoyloxy group, which is indispensable for potent irreversible inhibition of STS enzyme. Although the role of sulphamoyloxy group in these compounds remain unclear. The potency might have possibly arisen as a result from their absorption by the red blood cells, which protect these drugs from inactivation during passage through the liver and hence slowly released to cause the desired effect at the target site. It is possible that these sulphamates are acting as prodrugs of their respective parent compounds, which are relatively easily metabolised during their passage through the liver.

It is therefore interesting to establish some degree of structure-activity relationship for the 2-MeOEMATE class of compounds by synthesising structurally similar derivatives, such as 2-substituted estrone 3-sulphamide, 3-sulphonamide and 3-*S*-sulphamates, with nitrogen or sulphur bridging heteroatoms at the C-3 position instead of the O-atom in sulphamoyl group. These compounds with non- hydrolysable moieties at the C-3 position would provide an indication about the importance of the sulphamoyloxy group for the potent activity observed in the sulphamates. The sulphonamides are known to bind to carbonic anhydrase enzyme in the blood therefore might well show similar inhibitory activities to that of the sulphamates by being taken up into the blood cells. Most importantly, these compounds would offer useful information about the nature of the site where sulphamoyl group binds in tubulin. It will also reveal how the sulphamoyloxy group binds to tubulin to produce the irreversible inhibition as observed with EMATE and 2-MeOEMATE. Their increased binding affinity to the colchicine receptor might be the driving force behind the increase in potency observed or the binding pattern might be similar to that of sulphamoylation of sulphatase enzyme. Therefore, several novel sulphamate surrogates such as 2-methoxy and 2-ethylestrone-3-sulphamide, 3-sulphonamide and 3-*S*-sulphamate analogues were synthesised in this project and their synthetic procedures are reported here. The nature of inhibition of these compounds can only be validated once the biological activity results are made available, which is currently underway.

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APPENDIX

Appendix A

Biological testing

All the compounds submitted were tested for their biological activity at the Department of Endocrinology and Metabolic Medicine, St. Mary's Hospital Medical School, Imperial College, London, by Dr. A. Purohit and Ms. B. Malini.

Cell culture

MCF-7 breast cancer cells were maintained in minimal essential medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, 5% fetal bovine serum, 2 mM glutamine, nonessential amino acids and 0.075% sodium bicarbonate. For experiments, up to 30 replicate 25 cm^2 tissue culture flasks were seeded with about 1×10^5 cells/flasks using the medium described above. For testing the effect of inhibitor *in vitro* E1-STS activity, the cells were allowed to grow to 80% confluency and the medium was changed on every third day.

(A) *In vitro* estrone sulphatase (E1-STS) assay

(i) On cell monolayers[188,224]

Intact monolayers of MCF-7 cells in triplicate 25 cm^2 tissue culture flasks were washed with Earle's balanced salt solution and incubated for 20 h at 37 °C with [^{3}H]E1S (5 pmol, 7×10^5 dpm) in serum-free minimal essential medium (2.5 ml) with or without the synthetic analogues (0.25 - 25 nmol). Substrate and inhibitor were added to medium in ethanol, with the final concentration being <1%. After incubating, each flask was cooled and the medium (1 ml) was pipetted into separate tubes containing [^{14}C]E1 (7×10^3 dpm). The mixture was shaken vigorously for 30 s with toluene (5 ml). Preliminary experiments showed that >90% [^{14}C]E1 and <0.1% [^{3}H]E1S was removed from the aqueous phase by this treatment. A portion (2 ml) of the organic phase was removed and evaporated and the ^{3}H and ^{14}C content of the residue was determined by

scintillation spectrometry. The mass of E1S hydrolysed was calculated from the ^{3}H counts obtained (corrected for the volumes of the medium and organic phase used and for recovery of [^{14}C]E1 added) and the specific activity of the substrate. Each batch of experiments included incubation of microsomes prepared from a sulphatase positive human placenta (positive control) and the flasks without cells (to assess apparent non-enzymic hydrolysis of the substrate). The number of cell nuclei per flask was determined using a Coulter Counter after treating the cell monolayers with zaponin. Unless otherwise stated, the results are expressed as the means ± SD of the total product (estrone + estradiol) formed during the incubation period (3 - 4 h) and calculated for 10^6 cells.

(ii) On placental microsomes[188]

The ability of the compounds synthesised to inhibit steroid sulphatase activity was tested using a placental microsomal preparation from a sulphatase-positive human placenta from a normal term pregnancy. E1-STS activity was measured in the absence and presence of inhibitors using [6,7-^{3}H]E1S (4×10^5 dpm, NEN-Du Pont, Boston, MA) adjusted to a final concentration of 20 μM with unlabelled substrate. After incubation of the substrate inhibitor with placental microsomes (100 μg protein) at 37 °C for 30 min, the product formed was isolated from the mixture by extraction with toluene (4 ml). [4-^{14}C]E1 (7×10^3 dpm) was used to monitor procedural losses.

The ability of the tricyclic COUMATEs to inhibit STS activity was examined using placental microsomes. Supernatants prepared from homogenised rat liver tissues were used to test the potency of selected inhibitors *in vitro* following the same procedure.[188]

Statistics

Unpaired Student's *t* test was used to test the statistical significance of our findings. One flask in each batch was used to assess cell membrane status and viability using the trypan blue exclusion method.

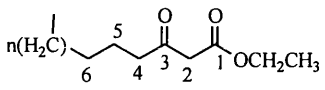
(B) *In vivo* studies[220]

Selected drugs were tested *in vivo* for their ability to inhibit STS activity. Female Wistar rats (200 - 250 g) were obtained from Harlan Olac (Bicester, Oxon, U.K). Groups of rats, with three rats in each group for each experiment, were tested p.o. with vehicle (propylene glycol) or drug (0.1 mg/Kg and 1.0 mg/Kg) with animals receiving a single dose. Animals were killed, using an approved procedure, 24 h after drug administration to assess the extent of STS inhibition. For this, samples of liver tissue were removed and immediately frozen on solid carbon dioxide and stored at –20 °C until assayed. For some animals samples of brain tissue were also collected to assess the extent of inhibition of STS in this tissue. Tissues were homogenised and after centrifugation to remove cell debris, aliquots of the supernatant were used for the STS assay.[224] For some animals, blood was also collected by cardiac puncture under anesthesia. White blood cells (WBCs) were obtained by centrifugation with Histopaque 1077 (Sigma, Poole, Dorset, U.K.) and used to assay STS activity.[266]

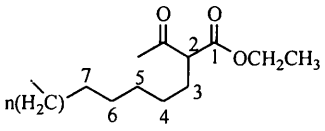
Appendix B

Numbering and nomenclature of compounds

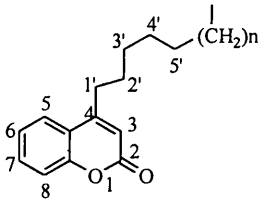
(1) Ethyl 3-oxoalkanoates



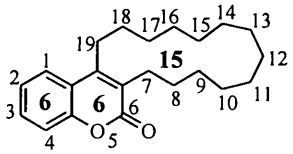
(2) Ethyl 2-acetylalkanoates



(3) 4-Alkylcoumarin Nucleus

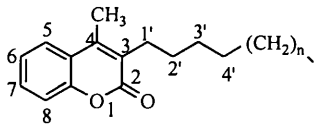


(4) Tricyclic Coumarin Nucleus

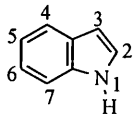


e.g. : 6-Oxo-8,9,10,11,12,13,14,15,16,17,18,19-dodecahydro-*7H*-cyclopentadeca-[c][1]-benzopyran (6615)

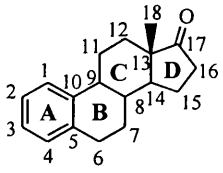
(5) 3-Alkyl-4-methylcoumarin Nucleus



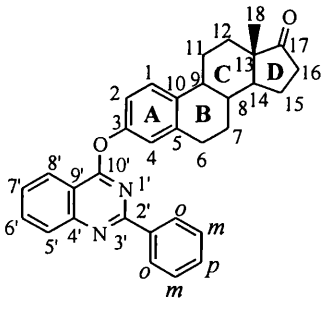
(6) Indole Nucleus



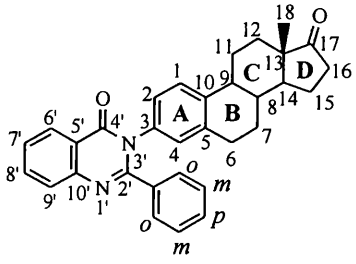
(7) Steroid Nucleus



(8) 3-(2'-Phenyl-4'-quinazolinyloxy) estrone



(9) 3-[4'-Oxo-2'-phenyl-3'(4*H*)-quinazolinyl]estrone



Appendix C

Log P Calculations

(A) 90: 10 Methanol : Water

Compounds	t_1	t_2	t_3	t_{av}	t_R-t_0	t_R-t_0/t_0	log P Known	log k_w Known	log K	S.D av	log k_w
Diethylstilbestrol	1.765	1.764	1.766	1.765	0.215	0.13870	5.07	4.16	-0.857893	0.001	4.3525
Nifedipine	1.819	1.817	1.82	1.81866	0.26866	0.17333	3.17	2.46	-0.761117	0.00152	2.3602
Testosterone	2.265	2.267	2.268	2.26666	0.71666	0.46236	3.29	2.74	-0.335014	0.00152	2.8089
Quinoline	1.93	1.926	1.924	1.92666	0.37666	0.24301	2.03	1.56	-0.614374	0.00305	1.3329
3-Br-quinoline	2.359	2.353	2.348	2.35333	0.80333	0.51827	3.03	2.54	-0.285435	0.00550	2.2028
3,5-Dichlorophenol	2.132	2.123	2.135	2.13	0.58	0.37419	3.68	3.4	-0.426903	0.00624	3.0781
Bifonazole	2.719	2.713	2.71	2.714	1.164	0.75096	4.77	4.37	-0.124378	0.00458	4.635
Clotrimazole	2.829	2.813	2.99	2.87733	1.32733	0.85634	5.2	4.13	-0.067351	0.09789	4.3719
Tolnaftate	2.848	2.865	2.843	2.852	1.302	0.84	5.4	4.55	-0.075720	0.01153	4.7549
Estradiol	1.905	1.905	1.906	1.90533	0.35533	0.22924	4.01	3.34	-0.639695	5.77350	3.0412
Diazepam	2.036	2.038	2.035	2.03633	0.48633	0.31376	2.79	2.63	-0.503397	0.00152	1.9688
3-Chlorophenol	1.791	1.793	1.792	1.792	0.242	0.15612	2.5	2.49	-0.806516	0.001	2.0917
665 Coumarin	1.831	1.832	1.838	1.83366	0.28366	0.18301	1.88		-0.737523	0.00378	
666 Coumarin	1.977	1.974	1.973	1.97466	0.42466	0.27397	2.3		-0.562283	0.00208	
667 Coumarin	2.018	2.018	2.019	2.01833	0.46833	0.30215	2.71		-0.519776	5.77350	
668 Coumarin	2.155	2.153	2.152	2.15333	0.60333	0.38924	3.13		-0.409774	0.00152	
669 Coumarin	2.405	2.408	2.408	2.407	0.857	0.55290	3.55		-0.257350	0.00173	
6610 Coumarin	2.768	2.754	2.772	2.76466	1.21466	0.78365	3.97		-0.105874	0.00945	
6611 Coumarin	3.365	3.362	3.362	3.363	1.813	1.16967	4.38		0.068066	0.00173	

6612 Coumarin	4.235	4.243	4.258	4.24533	2.69533	1.73892	4.8		0.240280	0.01167	
6613 Coumarin	5.367	5.29	5.248	5.30166	3.75166	2.42043	5.22		0.383892	0.06035	
6615 Coumarin	8.515	8.646	8.687	8.616	7.066	4.55870	6.05		0.658841	0.08983	
Coumarin	1.753	1.758	1.763	1.758	0.208	0.13419			-0.872268	0.005	
EMATE	1.733	1.739	1.739	1.737	0.187	0.12064	3.73		-0.918490	0.00346	
Estrone	1.946	1.995	2.007	1.98266	0.43266	0.27913	4.4		-0.554178	0.03231	
665 Coumate	1.61	1.613	1.611	1.61133	0.06133	0.03956	1.21		-1.402635	0.00152	
666 Coumate	1.669	1.668	1.667	1.668	0.118	0.07612	1.63		-1.118449	0.001	
667 Coumate	1.7	1.7	1.71	1.70333	0.15333	0.09892	2.04		-1.004695	0.00577	
668 Coumate	1.752	1.75	1.765	1.75566	0.20566	0.13268	2.46		-0.877167	0.00814	
669 Coumate	1.842	1.841	1.84	1.841	0.291	0.18774	2.88		-0.726438	0.001	
6610 Coumate	1.987	1.992	1.989	1.98933	0.43933	0.28344	3.29		-0.547537	0.00251	
6611 Coumate	2.23	2.28	2.21	2.24	0.69	0.44516	3.71		-0.351482	0.03605	
6612 Coumate	2.57	2.62	2.51	2.56666	1.01666	0.65591	4.13		-0.183153	0.05507	
6613 Coumate	3.066	3.069	3.054	3.063	1.513	0.97612	4.55		-0.010492	0.00793	
6615 Coumate	4.208	4.211	4.205	4.208	2.658	1.71483	5.38		0.234223	0.003	
Coumate	1.559	1.558	1.557	1.558	0.008	0.00516			-2.287241	0.001	

(B) 80 : 20 Methanol : Water

Diethylstilbestrol	2.326	2.329	2.327	2.32733	0.77733	0.50150			-0.299724	0.00152	
Nifedipine	2.163	2.151	2.155	2.15633	0.60633	0.39118			-0.407620	0.00611	
Testosterone	3.071	3.062	3.07	3.06766	1.51766	0.97913			-0.009155	0.00493	
Quinoline	2.158	2.166	2.159	2.161	0.611	0.39419			-0.404290	0.00435	
3-Br-quinoline	3.124	3.114	3.116	3.118	1.568	1.01161			0.005014	0.00529	
3,5-Dichlorophenol	3.005	2.981	3.015	3.00033	1.45033	0.93569			-0.028863	0.01747	
Bifonazole	5.437	5.464	5.442	5.44766	3.89766	2.51462			0.400472	0.01436	
Clotrimazole	5.591	5.544	5.541	5.55866	4.00866	2.58623			0.412668	0.02804	

Tolnaftate	6.193	6.159	6.17	6.174	4.624	2.98322			0.474686	0.01734	
Estradiol	2.581	2.567	2.566	2.57133	1.02133	0.65892			-0.181164	0.00838	
Diazepam	2.773	2.77	2.771	2.77133	1.22133	0.78795			-0.103497	0.00152	
3-Chlorophenol	2.142	2.125	2.133	2.13333	0.58333	0.37634			-0.424414	0.00850	
665 Coumarin	2.047	2.05	2.048	2.04833	0.49833	0.32150			-0.492811	0.00152	
666 Coumarin	2.354	2.355	2.355	2.35466	0.80466	0.51913			-0.284715	5.77350	
667 Coumarin	2.514	2.53	2.521	2.52166	0.97166	0.62688			-0.202814	0.00802	
668 Coumarin	2.892	2.88	2.89	2.88733	1.33733	0.86279			-0.064092	0.00642	
669 Coumarin	3.513	3.512	3.511	3.512	1.962	1.26580			0.102367	10.0000	
6610 Coumarin	4.532	4.53	4.531	4.531	2.981	1.92322			0.284030	0.001	
6611 Coumarin	6.458	6.457	6.45	6.455	4.905	3.16451			0.500307	0.00435	
6612 Coumarin	9.916	9.911	9.914	9.91366	8.36366	5.39591			0.732065	0.00251	
6613 Coumarin	15.207	15.2	15.211	15.206	13.656	8.81032			0.944991	0.00556	
6615 Coumarin	30.213	30.214	30.209	30.212	28.662	18.4916			1.266974	0.00264	
Coumarin	1.865	1.862	1.863	1.86333	0.31333	0.20215			-0.694325	0.00152	
EMATE	2.051	2.057	2.056	2.05466	0.50466	0.32559			-0.487327	0.00321	
Estrone	2.697	2.681	2.666	2.68133	1.13133	0.72989			-0.136741	0.01550	
665 Coumate	1.752	1.744	1.747	1.74766	0.19766	0.12752			-0.894398	0.00404	
666 Coumate	1.893	1.894	1.895	1.894	0.344	0.22193			-0.653773	10.0000	
667 Coumate	1.995	1.992	1.993	1.99333	0.44333	0.28602			-0.543601	0.00152	
668 Coumate	2.162	2.144	2.158	2.15466	0.60466	0.39010			-0.408815	0.00945	
669 Coumate	2.46	2.44	2.465	2.455	0.905	0.58387			-0.233683	0.01322	
6610 Coumate	2.936	2.941	2.929	2.93533	1.38533	0.89376			-0.048777	0.00602	
6611 Coumate	3.79	3.82	3.75	3.78666	2.23666	1.44301			0.159269	0.03511	
6612 Coumate	5.309	5.299	5.311	5.30633	3.75633	2.42344			0.384432	0.00642	
6613 Coumate	6.523	6.519	6.526	6.52266	4.97266	3.20817			0.506257	0.00351	
6615 Coumate	10.83	10.799	10.845	10.8246	9.27466	5.98365			0.776966	0.02345	
Coumate	1.636	1.63	1.638	1.63466	0.08466	0.05462			-1.262619	0.00416	

(C) 70 : 30 Methanol : Water

Diethylstilbestrol	4.653	4.652	4.655	4.65333	3.10333	2.00215			0.301496	0.00152	
Nifedipine	2.875	2.871	2.88	2.87533	1.32533	0.85505			-0.068006	0.00450	
Testosterone	5.16	5.161	5.116	5.14566	3.59566	2.31978			0.365447	0.02569	
Quinoline	2.568	2.568	2.578	2.57133	1.02133	0.65892			-0.181164	0.00577	
3-Br-quinoline	4.411	4.415	4.412	4.41266	2.86266	1.84688			0.266439	0.00208	
3,5-Dichlorophenol	5.088	4.986	5.01	5.028	3.478	2.24387			0.350997	0.05332	
Bifonazole	14.853	14.85	14.852	14.8516	13.3016	8.58172			0.933574	0.00152	
Clotrimazole	14.447	14.448	14.446	14.447	12.897	8.32064			0.920157	10.0000	
Tolnaftate	16.934	16.932	16.933	16.933	15.383	9.92451			0.996709	0.001	
Estradiol	3.867	3.865	3.867	3.86633	2.31633	1.49440			0.174469	0.00115	
Diazepam	3.221	3.237	3.248	3.23533	1.68533	1.08731			0.036354	0.01357	
3-Chlorophenol	2.606	2.61	2.599	2.605	1.055	0.68064			-0.167079	0.00556	
665 Coumarin	2.519	2.521	2.518	2.51933	0.96933	0.62537			-0.203858	0.00152	
666 Coumarin	3.202	3.2	3.199	3.20033	1.65033	1.06473			0.027239	0.00152	
667 Coumarin	3.788	3.782	3.779	3.783	2.233	1.44064			0.158557	0.00458	
668 Coumarin	4.759	4.748	4.761	4.756	3.206	2.06838			0.315631	0.007	
669 Coumarin	6.505	6.499	6.509	6.50433	4.95433	3.19634			0.504653	0.00503	
6610 Coumarin	9.772	9.769	9.771	9.77066	8.22066	5.30365			0.724575	0.00152	
6611 Coumarin	16.861	16.854	16.869	16.8613	15.3113	9.87827			0.994681	0.00750	
6612 Coumarin	33.954	33.955	33.951	33.9533	32.4033	20.9053			1.320257	0.00208	
6613 Coumarin	59.109	59.047	59.07	59.0753	57.5253	37.1131			1.569527	0.03134	
6615 Coumarin	77.85	78.217	76.98	77.6823	76.1323	49.1176			1.691237	0.63531	
Coumarin	2.098	2.099	2.1	2.099	0.549	0.35419			-0.450759	10.0000	
EMATE	3.182	3.174	3.125	3.16033	1.61033	1.03892			0.016584	0.03085	
Estrone	4.162	4.161	4.159	4.16066	2.61066	1.68430			0.226419	0.00152	
665 Coumate	2.018	2.022	2.02	2.02	0.47	0.30322			-0.518233	0.002	

666 Coumate	2.379	2.373	2.375	2.37566	0.82566	0.53268			-0.273526	0.00305	
667 Coumate	2.762	2.809	2.791	2.78733	1.23733	0.79827			-0.097844	0.02371	
668 Coumate	3.256	3.263	3.259	3.25933	1.70933	1.10279			0.042495	0.00351	
669 Coumate	4.258	4.252	4.256	4.25533	2.70533	1.74537			0.241889	0.00305	
6610 Coumate	6.145	6.144	6.146	6.145	4.595	2.96451			0.471953	0.001	
6611 Coumate	9.654	9.649	9.662	9.655	8.105	5.22903			0.718421	0.00655	
6612 Coumate	16.313	16.315	16.309	16.3123	14.7623	9.52408			0.978823	0.00305	
6613 Coumate	27.836	27.828	27.841	27.835	26.285	16.9580			1.229376	0.00655	
6615 Coumate	60.274	60.252	60.248	60.258	58.708	37.8761			1.578365	0.014	
Coumate	1.744	1.747	1.745	1.74533	0.19533	0.12602			-0.899555	0.00152	

(D) Determined log P values

Compounds	Mobile Phase	k	log k	log k_w	log P
Diethylstilbestrol	70%	2.0024	0.301550	4.16	**5.07**
	80%	0.50151	-0.299720		
	90%	0.1387	-0.857923		
Nifedipine	70%	0.85505	-0.068008	2.46	**3.17**
	80%	0.39118	-0.407623		
	90%	0.17333	-0.761126		
Testosterone	70%	2.31978	0.365446	2.74	**3.29**
	80%	0.97914	-0.009155		
	90%	0.46237	-0.335010		
Quinoline	70%	0.65892	-0.181167	1.56	**2.03**
	80%	0.39419	-0.404294		
	90%	0.24301	-0.614375		
3-Br-quinoline	70%	1.84688	0.266438	2.54	**3.03**
	80%	1.01161	0.005013		
	90%	0.51828	-0.285435		
3,5-Dichlorophenol	70%	2.2439	0.351003	3.4	**3.68**
	80%	0.94495	-0.024591		
	90%	0.3742	-0.426896		
Bifonazole	70%	8.5817	0.933573	4.37	**4.77**
	80%	2.51462	0.400472		
	90%	0.75097	-0.124377		
Clotrimazole	70%	8.32065	0.920157	4.13	**5.20**
	80%	2.58624	0.412668		
	90%	0.85634	-0.067353		
Tolnaftate	70%	9.9245	0.996708	4.55	**5.40**
	80%	2.98323	0.474686		
	90%	0.84	-0.075720		
Estradiol	70%	1.49441	0.174469	3.34	**4.01**
	80%	0.65892	-0.181167		
	90%	0.22925	-0.639690		
Diazepam	70%	1.0873	0.036349	2.63	**2.79**
	80%	0.788	-0.103473		
	90%	0.31376	-0.503402		
3-Chlorophenol	70%	0.68065	-0.167076	2.49	**2.50**
	80%	0.37634	-0.424419		
	90%	0.15613	-0.806513		
665 Coumarin	70%	0.6254	-0.203842	1.66	2.39
	80%	0.32151	-0.492805		
	90%	0.18301	-0.737525		
666 Coumarin	70%	1.0647	0.027227	2.08	2.79
	80%	0.51914	-0.284715		
	90%	0.27398	-0.562281		

667 Coumarin	70%	1.4406	0.158543	2.52	3.22
	80%	0.62688	-0.202815		
	90%	0.30215	-0.519777		
668 Coumarin	70%	2.0684	0.315634	2.84	3.52
	80%	0.8628	-0.064089		
	90%	0.38925	-0.409771		
669 Coumarin	70%	3.1963	0.504647	3.16	3.82
	80%	1.26581	0.102368		
	90%	0.5529	-0.257353		
6610 Coumarin	70%	2.9645	0.471951	3.62	4.26
	80%	1.92323	0.284031		
	90%	0.78366	-0.105872		
6611 Coumarin	70%	9.8783	0.994682	4.23	4.83
	80%	3.16452	0.500307		
	90%	1.16968	0.068067		
6612 Coumarin	70%	20.905	1.320250	5.08	5.64
	80%	5.39591	0.732064		
	90%	1.73892	0.240279		
6613 Coumarin	70%	37.113	1.569526	5.21	5.76
	80%	8.81032	0.944991		
	90%	2.42043	0.383892		
6615 Coumarin	70%	49.1176	1.691237	5.71	6.24
	80%	18.4916	1.266974		
	90%	4.55871	0.658841		
Coumarin	70%	0.35419	-0.450763	1.01	1.82
	80%	0.20215	-0.694326		
	90%	0.13419	-0.872279		
EMATE	70%	1.0389	0.016573	3.28	3.92
	80%	0.32559	-0.487328		
	90%	0.12065	-0.918472		
Estrone	70%	1.6843	0.226419	2.97	3.64
	80%	0.72989	-0.136742		
	90%	0.27914	-0.554177		
665 COUMATE	70%	0.3032	-0.518270	1.61	2.34
	80%	0.1275	-0.894489		
	90%	0.0396	-1.402304		
666 COUMATE	70%	0.5327	-0.273517	1.99	2.71
	80%	0.2219	-0.653842		
	90%	0.0761	-1.118615		
667 COUMATE	70%	0.7983	-0.097833	2.46	3.15
	80%	0.286	-0.543633		
	90%	0.0989	-1.004803		
668 COUMATE	70%	1.1028	0.042496	2.79	3.46
	80%	0.3901	-0.408824		
	90%	0.1327	-0.877129		

669 COUMATE	70%	1.7454	0.241894	3.09	3.75
	80%	0.5839	-0.233661		
	90%	0.1877	-0.726535		
6610 COUMATE	70%	2.9847	0.474900	3.26	3.92
	80%	0.8938	-0.048759		
	90%	0.2834	-0.547600		
6611 COUMATE	70%	5.229	0.718418	4.19	4.80
	80%	1.443	0.159266		
	90%	0.4452	-0.351444		
6612 COUMATE	70%	9.5241	0.978823	5.02	5.58
	80%	2.4234	0.384425		
	90%	0.6559	-0.183162		
6613 COUMATE	70%	16.958	1.229374	5.18	5.74
	80%	3.2082	0.506261		
	90%	0.9761	-0.010505		
6615 COUMATE	70%	37.876	1.578364	5.66	6.22
	80%	5.9837	0.776969		
	90%	1.7148	0.234213		
COUMATE	70%	0.126	-0.899629	1.01	1.77
	80%	0.546	-0.262807		
	90%	0.0052	-2.283996		

Key : — Known log P values from the literature

Appendix D

Table 1. Crystal data and structure refinement for 669 COUMATE (119)

Identification code	k00bvlp2
Empirical formula	C17.50 H22.50 N O5 S
Formula weight	358.92
Temperature	170(2) K
Wavelength	0.71070 Å
Crystal system	Monoclinic
Space group	$P2_1/n$
Unit cell dimensions	a = 9.9960(2)Å α = 90°
	b = 18.5330(4)Å β = 98.4970(13)°
	c = 18.9890(4)Å γ = 90°
Volume	3479.21(13) $Å^3$
Z	8
Density (calculated)	1.370 Mg/m^3
Absorption coefficient	0.214 mm^{-1}
F(000)	1524
Crystal size	0.25 x 0.25 x 0.10 mm
Theta range for data collection	3.53 to 27.89 °.
Index ranges	0<=h<=13; -24<=k<=24; -24<=l<=24
Reflections collected	34928
Independent reflections	8111 [R(int) = 0.0517]
Reflections observed (>2σ)	6956
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	8111 / 0 / 459
Goodness-of-fit on F^2	0.913
Final R indices [I>2σ(I)]	R_1 = 0.0757 wR_2 = 0.2195
R indices (all data)	R_1 = 0.0947 wR_2 = 0.2330
Largest diff. peak and hole	0.330 and -0.364 $e.Å^{-3}$

Notes:

Asymmetric unit = 2 molecules of compound plus ½ molecule of hexane. Latter straddles space group inversion centre.

Intra/intermolecular hydrogen bonding contacts involving NH_2 hydrogens. Distances/angles summarised below.

Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg.

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
N1-H1A	0.814	2.244	160.43	3.023	O10
N1-H1A	0.814	2.629	144.54	3.325	O9
N1-H1B	0.888	1.969	165.15	2.836	O5 [x+1/2, -y+1/2, z+1/2]
N2-H2A	0.784	2.412	175.29	3.194	O4
N2-H2B	0.824	2.065	166.06	2.871	O1 [x-1/2, -y+1/2, z-1/2]

Table 2 : Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for 1.U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

Atom	x	y	z	U(eq)
S(1)	8305(1)	2918(1)	4407(1)	27(1)
S(2)	7482(1)	2649(1)	1281(1)	27(1)
O(1)	9603(2)	2786(2)	4808(1)	37(1)
O(2)	8180(3)	3080(2)	3667(1)	35(1)
O(3)	7561(2)	2156(1)	4487(1)	29(1)
O(4)	4627(2)	1785(1)	2319(1)	22(1)
O(5)	3405(2)	1606(1)	1282(1)	31(1)
O(6)	8333(3)	2719(2)	750(2)	42(1)
O(7)	8035(2)	2588(2)	2011(1)	34(1)
O(8)	6602(2)	3381(1)	1178(1)	31(1)
O(9)	5129(2)	3748(1)	3412(1)	25(1)
O(10)	4600(3)	3934(2)	4476(1)	36(1)
N(1)	7550(3)	3514(2)	4795(2)	33(1)
N(2)	6464(3)	1991(2)	1093(2)	32(1)
C(1)	6245(3)	2072(2)	4113(2)	25(1)
C(2)	6073(3)	1958(2)	3387(2)	22(1)
C(3)	4756(3)	1880(2)	3047(2)	20(1)
C(4)	3379(3)	1699(2)	1916(2)	22(1)
C(5)	2170(3)	1743(2)	2259(2)	23(1)
C(6)	867(3)	1657(2)	1749(2)	28(1)
C(7)	501(4)	865(2)	1547(2)	37(1)
C(8)	-791(4)	592(2)	1815(2)	39(1)
C(9)	-909(4)	741(2)	2589(2)	39(1)
C(10)	338(4)	567(2)	3140(2)	38(1)
C(11)	705(4)	1173(2)	3686(2)	33(1)
C(12)	1066(3)	1896(2)	3360(2)	26(1)
C(13)	2279(3)	1841(2)	2975(2)	22(1)
C(14)	3622(3)	1899(2)	3394(2)	21(1)
C(15)	3869(3)	2002(2)	4137(2)	26(1)
C(16)	5165(3)	2090(2)	4497(2)	28(1)
C(17)	5555(3)	3456(2)	1596(2)	26(1)
C(18)	5861(3)	3579(2)	2318(2)	24(1)
C(19)	4782(3)	3648(2)	2695(2)	22(1)
C(20)	4163(3)	3834(2)	3856(2)	27(1)
C(21)	2736(3)	3778(2)	3544(2)	25(1)
C(22)	1791(4)	3870(2)	4090(2)	29(1)
C(23)	1564(4)	4675(2)	4267(2)	37(1)
C(24)	131(4)	4957(2)	4040(2)	42(1)
C(25)	-505(4)	4790(2)	3277(2)	38(1)
C(26)	365(4)	4944(2)	2687(2)	36(1)
C(27)	366(4)	4324(2)	2154(2)	32(1)

C(28)	920(3)	3612(2)	2487(2)	26(1)
C(29)	2378(3)	3671(2)	2837(2)	24(1)
C(30)	3432(3)	3610(2)	2383(2)	22(1)
C(31)	3186(3)	3501(2)	1644(2)	27(1)
C(32)	4231(4)	3423(2)	1247(2)	28(1)
C(101)	5547(4)	-24(2)	4764(2)	46(1)
C(102)	6898(5)	284(3)	5099(3)	50(1)
C(103)	7999(6)	200(3)	4633(3)	68(2)

Table 3 : Bond lengths [Å] and angles [°] for 1.

S(1)-O(1)	1.424(3)
S(1)-O(2)	1.424(2)
S(1)-N(1)	1.581(4)
S(1)-O(3)	1.614(3)
S(2)-O(6)	1.418(3)
S(2)-O(7)	1.420(2)
S(2)-N(2)	1.595(3)
S(2)-O(8)	1.613(3)
O(3)-C(1)	1.407(4)
O(4)-C(4)	1.373(4)
O(4)-C(3)	1.379(4)
O(5)-C(4)	1.219(4)
O(8)-C(17)	1.409(4)
O(9)-C(19)	1.367(4)
O(9)-C(20)	1.382(4)
O(10)-C(20)	1.207(4)
C(1)-C(2)	1.382(4)
C(1)-C(16)	1.390(5)
C(2)-C(3)	1.386(4)
C(3)-C(14)	1.394(4)
C(4)-C(5)	1.457(4)
C(5)-C(13)	1.361(4)
C(5)-C(6)	1.512(4)
C(6)-C(7)	1.548(5)
C(7)-C(8)	1.540(5)
C(8)-C(9)	1.517(6)
C(9)-C(10)	1.538(5)
C(10)-C(11)	1.536(6)
C(11)-C(12)	1.541(5)
C(12)-C(13)	1.509(4)
C(13)-C(14)	1.459(4)
C(14)-C(15)	1.409(4)
C(15)-C(16)	1.382(5)
C(17)-C(18)	1.380(5)
C(17)-C(32)	1.390(5)
C(18)-C(19)	1.386(4)
C(19)-C(30)	1.394(4)
C(20)-C(21)	1.466(5)
C(21)-C(29)	1.353(5)
C(21)-C(22)	1.512(4)
C(22)-C(23)	1.552(5)
C(23)-C(24)	1.526(6)
C(24)-C(25)	1.524(6)
C(25)-C(26)	1.544(5)
C(26)-C(27)	1.532(5)
C(27)-C(28)	1.531(5)

C(28)-C(29)	1.514(4)
C(29)-C(30)	1.461(4)
C(30)-C(31)	1.403(5)
C(31)-C(32)	1.383(5)
C(101)-C(101)#1	1.515(9)
C(101)-C(102)	1.516(6)
C(102)-C(103)	1.519(7)
O(1)-S(1)-O(2)	120.25(16)
O(1)-S(1)-N(1)	109.28(17)
O(2)-S(1)-N(1)	109.49(18)
O(1)-S(1)-O(3)	101.32(15)
O(2)-S(1)-O(3)	107.67(14)
N(1)-S(1)-O(3)	108.00(16)
O(6)-S(2)-O(7)	120.87(17)
O(6)-S(2)-N(2)	109.96(19)
O(7)-S(2)-N(2)	107.50(18)
O(6)-S(2)-O(8)	102.23(15)
O(7)-S(2)-O(8)	108.17(14)
N(2)-S(2)-O(8)	107.33(15)
C(1)-O(3)-S(1)	117.3(2)
C(4)-O(4)-C(3)	121.1(2)
C(17)-O(8)-S(2)	116.9(2)
C(19)-O(9)-C(20)	121.7(3)
C(2)-C(1)-C(16)	122.4(3)
C(2)-C(1)-O(3)	119.3(3)
C(16)-C(1)-O(3)	118.3(3)
C(1)-C(2)-C(3)	116.8(3)
O(4)-C(3)-C(2)	115.0(3)
O(4)-C(3)-C(14)	121.0(3)
C(2)-C(3)-C(14)	124.1(3)
O(5)-C(4)-O(4)	114.6(3)
O(5)-C(4)-C(5)	126.1(3)
O(4)-C(4)-C(5)	119.2(3)
C(13)-C(5)-C(4)	120.4(3)
C(13)-C(5)-C(6)	126.1(3)
C(4)-C(5)-C(6)	113.6(3)
C(5)-C(6)-C(7)	114.0(3)
C(8)-C(7)-C(6)	114.1(3)
C(9)-C(8)-C(7)	116.6(3)
C(8)-C(9)-C(10)	116.7(3)
C(11)-C(10)-C(9)	113.3(3)
C(10)-C(11)-C(12)	114.3(3)
C(13)-C(12)-C(11)	112.9(3)
C(5)-C(13)-C(14)	119.1(3)
C(5)-C(13)-C(12)	122.8(3)
C(14)-C(13)-C(12)	118.1(3)
C(3)-C(14)-C(15)	116.2(3)

C(3)-C(14)-C(13)	119.2(3)
C(15)-C(14)-C(13)	124.5(3)
C(16)-C(15)-C(14)	121.7(3)
C(15)-C(16)-C(1)	118.8(3)
C(18)-C(17)-C(32)	122.4(3)
C(18)-C(17)-O(8)	120.1(3)
C(32)-C(17)-O(8)	117.5(3)
C(17)-C(18)-C(19)	117.0(3)
O(9)-C(19)-C(18)	115.2(3)
O(9)-C(19)-C(30)	121.2(3)
C(18)-C(19)-C(30)	123.6(3)
O(10)-C(20)-O(9)	115.3(3)
O(10)-C(20)-C(21)	126.5(3)
O(9)-C(20)-C(21)	118.1(3)
C(29)-C(21)-C(20)	120.7(3)
C(29)-C(21)-C(22)	126.7(3)
C(20)-C(21)-C(22)	112.6(3)
C(21)-C(22)-C(23)	112.5(3)
C(24)-C(23)-C(22)	115.4(3)
C(25)-C(24)-C(23)	116.1(3)
C(24)-C(25)-C(26)	116.8(3)
C(27)-C(26)-C(25)	113.4(3)
C(28)-C(27)-C(26)	114.1(3)
C(29)-C(28)-C(27)	112.7(3)
C(21)-C(29)-C(30)	119.3(3)
C(21)-C(29)-C(28)	122.9(3)
C(30)-C(29)-C(28)	117.8(3)
C(19)-C(30)-C(31)	116.7(3)
C(19)-C(30)-C(29)	118.8(3)
C(31)-C(30)-C(29)	124.5(3)
C(32)-C(31)-C(30)	121.7(3)
C(31)-C(32)-C(17)	118.6(3)
C(101)#1-C(101)-C(102)	114.0(4)
C(101)-C(102)-C(103)	113.2(4)

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,-y,-z+1

Table 4 : Anisotropic displacement parameters ($Å^2$ x 10^3) for 1. The anisotropic displacement factor exponent takes the form: -2 gpi^2 [h^2 a^{*2} U11 + ... + 2 h k a* b* U

Atom	U11	U22	U33	U23	U13	U12
S(1)	21(1)	42(1)	18(1)	4(1)	-1(1)	-6(1)
S(2)	19(1)	41(1)	21(1)	2(1)	6(1)	5(1)
O(1)	21(1)	61(2)	28(1)	3(1)	-2(1)	-5(1)
O(2)	31(1)	53(2)	21(1)	7(1)	1(1)	-13(1)
O(3)	24(1)	37(1)	24(1)	5(1)	-5(1)	-1(1)
O(4)	19(1)	29(1)	17(1)	-2(1)	4(1)	-1(1)
O(5)	28(1)	45(2)	20(1)	-4(1)	2(1)	-1(1)
O(6)	33(1)	62(2)	36(2)	4(1)	21(1)	5(1)
O(7)	25(1)	52(2)	24(1)	-3(1)	0(1)	10(1)
O(8)	31(1)	37(1)	27(1)	9(1)	12(1)	7(1)
O(9)	22(1)	32(1)	21(1)	-2(1)	2(1)	3(1)
O(10)	32(1)	50(2)	26(1)	-6(1)	3(1)	4(1)
N(1)	31(2)	42(2)	25(2)	1(1)	-5(1)	-1(1)
N(2)	28(2)	41(2)	26(2)	-2(1)	2(1)	5(1)
C(1)	22(2)	26(2)	26(2)	4(1)	1(1)	-1(1)
C(2)	20(1)	25(2)	22(2)	2(1)	4(1)	0(1)
C(3)	22(1)	22(1)	17(1)	0(1)	3(1)	0(1)
C(4)	23(2)	24(2)	20(1)	1(1)	1(1)	0(1)
C(5)	19(1)	21(1)	28(2)	2(1)	4(1)	0(1)
C(6)	20(2)	32(2)	30(2)	2(1)	-1(1)	2(1)
C(7)	26(2)	42(2)	41(2)	-14(2)	-4(2)	1(2)
C(8)	27(2)	30(2)	58(3)	-8(2)	-1(2)	-2(1)
C(9)	30(2)	31(2)	55(2)	2(2)	5(2)	-7(2)
C(10)	36(2)	30(2)	47(2)	5(2)	6(2)	-4(2)
C(11)	28(2)	37(2)	38(2)	6(2)	13(2)	-5(1)
C(12)	22(2)	26(2)	33(2)	-2(1)	8(1)	-1(1)
C(13)	21(1)	20(1)	24(2)	1(1)	6(1)	-2(1)
C(14)	22(1)	22(1)	20(1)	0(1)	5(1)	0(1)
C(15)	25(2)	31(2)	22(2)	-1(1)	8(1)	-3(1)
C(16)	29(2)	35(2)	19(1)	2(1)	5(1)	-3(1)
C(17)	25(2)	28(2)	26(2)	3(1)	8(1)	4(1)
C(18)	19(1)	24(2)	28(2)	2(1)	2(1)	2(1)
C(19)	24(2)	21(1)	22(2)	0(1)	3(1)	2(1)
C(20)	28(2)	28(2)	25(2)	1(1)	8(1)	1(1)
C(21)	25(2)	20(1)	30(2)	3(1)	6(1)	2(1)
C(22)	29(2)	33(2)	28(2)	5(1)	12(1)	1(1)
C(23)	35(2)	41(2)	38(2)	-9(2)	14(2)	1(2)
C(24)	38(2)	42(2)	51(2)	-3(2)	22(2)	7(2)
C(25)	29(2)	36(2)	51(2)	-4(2)	13(2)	10(2)
C(26)	40(2)	26(2)	43(2)	3(2)	9(2)	6(2)
C(27)	24(2)	34(2)	38(2)	3(2)	1(1)	5(1)
C(28)	18(1)	29(2)	31(2)	-2(1)	2(1)	0(1)

C(29)	19(1)	20(1)	33(2)	2(1)	5(1)	3(1)
C(30)	22(1)	21(1)	24(2)	1(1)	2(1)	3(1)
C(31)	24(2)	25(2)	30(2)	0(1)	-2(1)	4(1)
C(32)	32(2)	30(2)	22(2)	0(1)	1(1)	7(1)
C(101)	52(3)	48(2)	36(2)	-5(2)	5(2)	-7(2)
C(102)	52(3)	47(2)	52(3)	0(2)	6(2)	-1(2)
C(103)	58(3)	65(3)	87(4)	-8(3)	27(3)	-9(3)

Table 5 : Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for 1.

Atom	X	y	z	U(eq)
H(1A)	6810(50)	3650(30)	4610(30)	50(14)
H(1B)	7680(50)	3430(20)	5260(30)	45(13)
H(2A)	6050(50)	1920(30)	1400(30)	41(13)
H(2B)	6040(60)	2030(30)	690(30)	58(16)
H(2)	6822	1933	3132	27
H(6A)	944	1930	1308	34
H(6B)	120	1873	1966	34
H(7A)	382	818	1022	45
H(7B)	1266	551	1746	45
H(8A)	-1580	813	1516	47
H(8B)	-852	64	1737	47
H(9A)	-1683	460	2713	46
H(9B)	-1128	1258	2635	46
H(10A)	162	119	3395	45
H(10B)	1120	476	2887	45
H(11A)	1483	1014	4035	40
H(11B)	-69	1252	3948	40
H(12A)	280	2065	3022	32
H(12B)	1252	2259	3744	32
H(15)	3127	2010	4397	31
H(16)	5314	2162	4998	33
H(18)	6771	3615	2547	29
H(22A)	2170	3614	4532	35
H(22B)	908	3646	3907	35
H(23A)	1802	4742	4787	45
H(23B)	2194	4973	4033	45
H(24A)	-458	4753	4366	51
H(24B)	141	5487	4104	51
H(25A)	-1352	5072	3171	46
H(25B)	-757	4273	3252	46
H(26A)	22	5386	2427	43
H(26B)	1308	5039	2911	43
H(27A)	-572	4245	1915	39
H(27B)	915	4469	1784	39
H(28A)	357	3458	2847	31
H(28B)	851	3237	2112	31
H(31)	2279	3480	1410	32
H(32)	4049	3349	747	34
H(10C)	5240	237	4315	55
H(10D)	5675	-537	4644	55
H(10E)	7188	39	5560	60
H(10F)	6783	803	5198	60
H(10G)	8846	405	4878	102

H(10H)	7732	454	4181	102
H(10I)	8130	-313	4539	102

Table 1. Crystal data and structure refinement for 6610 COUMATE (122)

Identification code	k00bvlp3
Empirical formula	C18.50 H24.50 N O5 S
Formula weight	372.95
Temperature	170(2) K
Wavelength	0.71070 Å
Crystal system	Monoclinic
Space group	$P2_1/n$
Unit cell dimensions	a = 9.9030(2)Å α = 90°
	b = 19.3450(3)Å β = 97.6790(10)°
	c = 19.3020(3)Å γ = 90°
Volume	3664.59(11) $Å^3$
Z	8
Density (calculated)	1.352 Mg/m^3
Absorption coefficient	0.206 mm^{-1}
F(000)	1588
Crystal size	0.30 x 0.20 x 0.20 mm
Theta range for data collection	3.57 to 27.50 °.
Index ranges	0<=h<=12; -25<=k<=25; -25<=l<=24
Reflections collected	48896
Independent reflections	8389 [R(int) = 0.0563]
Reflections observed (>2σ)	6292
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	8389 / 0 / 477
Goodness-of-fit on F^2	1.433
Final R indices [I>2σ(I)]	R_1 = 0.0607 wR_2 = 0.1898
R indices (all data)	R_1 = 0.0842 wR_2 = 0.2049
Largest diff. peak and hole	1.023 and -0.605 $e.Å^{-3}$

Notes:

Asymmetric unit = 2 molecules of compound plus ½ molecule of hexane. Latter straddles space group inversion centre. Intra/intermolecular hydrogen bonding contacts involving NH_2 hydrogens. Distances/angles summarised below.

Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg.

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
N1-H1A	0.711	2.154	165.73	2.849	O5 [x-1/2, -y+1/2, z-1/2]
N1-H1B	0.836	2.211	152.95	2.980	O10
N1-H1B	0.836	2.588	153.38	3.356	O9
N2-H2A	0.744	2.551	169.19	3.284	O4
N2-H2A	0.744	2.587	130.47	3.122	O5
N2-H2B	1.018	1.919	158.21	2.889	O2 [x+1/2, -y+1/2, z+1/2]

Table 2 : Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for 1.U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

Atom	x	y	z	U(eq)
S(1)	1653(1)	2264(1)	693(1)	29(1)
S(2)	2470(1)	2295(1)	3828(1)	32(1)
O(1)	1743(2)	2081(1)	1409(1)	37(1)
O(2)	367(2)	2416(1)	299(1)	38(1)
O(3)	2433(2)	2991(1)	642(1)	31(1)
O(4)	5300(2)	3258(1)	2797(1)	27(1)
O(5)	6476(2)	3268(1)	3834(1)	33(1)
O(6)	1927(2)	2407(1)	3119(1)	43(1)
O(7)	1606(2)	2188(1)	4345(1)	49(1)
O(8)	3352(2)	1594(1)	3872(1)	37(1)
O(9)	4821(2)	1367(1)	1647(1)	32(1)
O(10)	5341(2)	1312(1)	584(1)	43(1)
N(1)	2409(3)	1703(1)	296(1)	37(1)
N(2)	3495(2)	2908(1)	4062(2)	37(1)
C(1)	3749(2)	3056(1)	1023(1)	28(1)
C(2)	3887(2)	3142(1)	1731(1)	27(1)
C(3)	5204(2)	3200(1)	2083(1)	24(1)
C(4)	6537(2)	3262(1)	3210(1)	26(1)
C(5)	7782(2)	3250(1)	2882(1)	26(1)
C(6)	9066(2)	3204(1)	3401(1)	32(1)
C(7)	9470(3)	3835(1)	3865(1)	36(1)
C(8)	9723(3)	4519(2)	3523(2)	51(1)
C(9)	10970(3)	4610(2)	3180(2)	66(1)
C(10)	10945(3)	4295(2)	2487(2)	81(1)
C(11)	9665(3)	4429(1)	1907(2)	50(1)
C(12)	9330(3)	3816(1)	1421(1)	36(1)
C(13)	8941(2)	3150(1)	1787(1)	29(1)
C(14)	7709(2)	3211(1)	2170(1)	25(1)
C(15)	6368(2)	3187(1)	1750(1)	24(1)
C(16)	6150(2)	3109(1)	1017(1)	28(1)
C(17)	4861(2)	3042(1)	656(1)	30(1)
C(18)	4406(2)	1546(1)	3449(1)	30(1)
C(19)	4096(2)	1478(1)	2740(1)	29(1)
C(20)	5181(2)	1420(1)	2357(1)	26(1)
C(21)	5795(3)	1337(1)	1203(1)	31(1)
C(22)	7231(2)	1355(1)	1488(1)	29(1)
C(23)	8181(3)	1389(1)	938(1)	37(1)
C(24)	8265(3)	753(1)	464(1)	40(1)
C(25)	8648(3)	66(1)	808(2)	45(1)
C(26)	10097(4)	-54(2)	1149(2)	69(1)
C(27)	10520(3)	261(2)	1824(2)	65(1)
C(28)	9621(3)	155(1)	2420(2)	44(1)

C(29)	9633(3)	775(1)	2896(1)	36(1)
C(30)	9067(2)	1444(1)	2533(1)	34(1)
C(31)	7607(2)	1396(1)	2191(1)	28(1)
C(32)	6540(2)	1427(1)	2654(1)	26(1)
C(33)	6788(3)	1489(1)	3382(1)	31(1)
C(34)	5735(3)	1548(1)	3782(1)	33(1)
C(101)	1982(5)	4823(2)	391(3)	104(2)
C(102)	3073(5)	4744(2)	-85(2)	84(1)
C(103)	4430(4)	5028(2)	222(2)	71(1)

Table 3 : Bond lengths [Å] and angles [°] for 1.

S(1)-O(1)	1.4180(18)
S(1)-O(2)	1.4237(17)
S(1)-N(1)	1.576(2)
S(1)-O(3)	1.6141(17)
S(2)-O(7)	1.4146(19)
S(2)-O(6)	1.4187(19)
S(2)-N(2)	1.587(2)
S(2)-O(8)	1.6086(17)
O(3)-C(1)	1.413(3)
O(4)-C(4)	1.369(3)
O(4)-C(3)	1.373(3)
O(5)-C(4)	1.215(3)
O(8)-C(18)	1.412(3)
O(9)-C(20)	1.373(3)
O(9)-C(21)	1.374(3)
O(10)-C(21)	1.220(3)
C(1)-C(2)	1.366(3)
C(1)-C(17)	1.388(3)
C(2)-C(3)	1.392(3)
C(3)-C(15)	1.394(3)
C(4)-C(5)	1.459(3)
C(5)-C(14)	1.369(3)
C(5)-C(6)	1.512(3)
C(6)-C(7)	1.535(3)
C(7)-C(8)	1.515(4)
C(8)-C(9)	1.487(4)
C(9)-C(10)	1.467(5)
C(10)-C(11)	1.597(5)
C(11)-C(12)	1.520(4)
C(12)-C(13)	1.543(3)
C(13)-C(14)	1.514(3)
C(14)-C(15)	1.461(3)
C(15)-C(16)	1.410(3)
C(16)-C(17)	1.377(3)
C(18)-C(19)	1.367(3)
C(18)-C(34)	1.386(3)
C(19)-C(20)	1.389(3)
C(20)-C(32)	1.391(3)
C(21)-C(22)	1.454(4)
C(22)-C(31)	1.361(3)
C(22)-C(23)	1.510(3)
C(23)-C(24)	1.543(3)
C(24)-C(25)	1.511(4)
C(25)-C(26)	1.514(5)
C(26)-C(27)	1.447(5)
C(27)-C(28)	1.560(4)

C(28)-C(29)	1.510(4)
C(29)-C(30)	1.542(3)
C(30)-C(31)	1.510(3)
C(31)-C(32)	1.474(3)
C(32)-C(33)	1.398(3)
C(33)-C(34)	1.383(4)
C(101)-C(102)	1.518(6)
C(102)-C(103)	1.498(6)
C(103)-C(103)#1	1.510(8)
O(1)-S(1)-O(2)	120.52(11)
O(1)-S(1)-N(1)	109.25(13)
O(2)-S(1)-N(1)	109.33(13)
O(1)-S(1)-O(3)	108.02(10)
O(2)-S(1)-O(3)	100.92(10)
N(1)-S(1)-O(3)	107.94(11)
O(7)-S(2)-O(6)	121.09(13)
O(7)-S(2)-N(2)	109.40(14)
O(6)-S(2)-N(2)	107.83(14)
O(7)-S(2)-O(8)	102.43(11)
O(6)-S(2)-O(8)	108.22(10)
N(2)-S(2)-O(8)	107.02(11)
C(1)-O(3)-S(1)	117.43(14)
C(4)-O(4)-C(3)	121.40(18)
C(18)-O(8)-S(2)	117.61(14)
C(20)-O(9)-C(21)	120.97(19)
C(2)-C(1)-C(17)	122.2(2)
C(2)-C(1)-O(3)	119.6(2)
C(17)-C(1)-O(3)	118.2(2)
C(1)-C(2)-C(3)	117.4(2)
O(4)-C(3)-C(2)	115.5(2)
O(4)-C(3)-C(15)	120.9(2)
C(2)-C(3)-C(15)	123.5(2)
O(5)-C(4)-O(4)	114.7(2)
O(5)-C(4)-C(5)	125.9(2)
O(4)-C(4)-C(5)	119.4(2)
C(14)-C(5)-C(4)	120.1(2)
C(14)-C(5)-C(6)	126.1(2)
C(4)-C(5)-C(6)	113.5(2)
C(5)-C(6)-C(7)	118.1(2)
C(8)-C(7)-C(6)	119.1(2)
C(9)-C(8)-C(7)	119.5(3)
C(10)-C(9)-C(8)	116.3(3)
C(9)-C(10)-C(11)	119.1(3)
C(12)-C(11)-C(10)	113.2(2)
C(11)-C(12)-C(13)	114.6(2)
C(14)-C(13)-C(12)	115.17(19)
C(5)-C(14)-C(15)	118.8(2)

C(5)-C(14)-C(13)	123.9(2)
C(15)-C(14)-C(13)	117.3(2)
C(3)-C(15)-C(16)	116.1(2)
C(3)-C(15)-C(14)	119.3(2)
C(16)-C(15)-C(14)	124.5(2)
C(17)-C(16)-C(15)	121.7(2)
C(16)-C(17)-C(1)	119.0(2)
C(19)-C(18)-C(34)	122.5(2)
C(19)-C(18)-O(8)	120.1(2)
C(34)-C(18)-O(8)	117.4(2)
C(18)-C(19)-C(20)	117.1(2)
O(9)-C(20)-C(19)	115.0(2)
O(9)-C(20)-C(32)	121.4(2)
C(19)-C(20)-C(32)	123.6(2)
O(10)-C(21)-O(9)	114.4(2)
O(10)-C(21)-C(22)	125.8(2)
O(9)-C(21)-C(22)	119.8(2)
C(31)-C(22)-C(21)	120.1(2)
C(31)-C(22)-C(23)	125.8(2)
C(21)-C(22)-C(23)	113.9(2)
C(22)-C(23)-C(24)	118.0(2)
C(25)-C(24)-C(23)	118.1(2)
C(24)-C(25)-C(26)	119.5(3)
C(27)-C(26)-C(25)	118.1(3)
C(26)-C(27)-C(28)	118.9(3)
C(29)-C(28)-C(27)	112.7(3)
C(28)-C(29)-C(30)	114.7(2)
C(31)-C(30)-C(29)	114.5(2)
C(22)-C(31)-C(32)	119.0(2)
C(22)-C(31)-C(30)	123.9(2)
C(32)-C(31)-C(30)	117.0(2)
C(20)-C(32)-C(33)	116.5(2)
C(20)-C(32)-C(31)	118.8(2)
C(33)-C(32)-C(31)	124.7(2)
C(34)-C(33)-C(32)	121.6(2)
C(33)-C(34)-C(18)	118.7(2)
C(103)-C(102)-C(101)	113.4(4)
C(102)-C(103)-C(103)#1	116.6(4)

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,-y+1,-z

Table 4 : Anisotropic displacement parameters ($Å^2 \times 10^3$) for 1. The anisotropic displacement factor exponent takes the form: $-2\ gpi^2\ [\ h^2\ a^{*2}\ U11 + ... + 2\ h\ k\ a^*\ b^*\ U$

Atom	U11	U22	U33	U23	U13	U12
S(1)	22(1)	43(1)	23(1)	1(1)	1(1)	-4(1)
S(2)	21(1)	45(1)	30(1)	-1(1)	7(1)	3(1)
O(1)	33(1)	54(1)	24(1)	5(1)	2(1)	-11(1)
O(2)	21(1)	60(1)	30(1)	-1(1)	-2(1)	-2(1)
O(3)	24(1)	39(1)	28(1)	4(1)	-3(1)	-2(1)
O(4)	21(1)	37(1)	23(1)	-3(1)	4(1)	-2(1)
O(5)	31(1)	44(1)	24(1)	-2(1)	3(1)	-4(1)
O(6)	28(1)	67(1)	31(1)	-9(1)	-5(1)	13(1)
O(7)	38(1)	66(1)	49(1)	2(1)	25(1)	2(1)
O(8)	36(1)	42(1)	36(1)	7(1)	18(1)	7(1)
O(9)	28(1)	39(1)	28(1)	-2(1)	4(1)	5(1)
O(10)	46(1)	54(1)	28(1)	-1(1)	4(1)	12(1)
N(1)	36(1)	48(1)	26(1)	-4(1)	-3(1)	3(1)
N(2)	29(1)	44(1)	38(1)	-2(1)	5(1)	3(1)
C(1)	23(1)	30(1)	28(1)	1(1)	1(1)	-2(1)
C(2)	22(1)	32(1)	28(1)	-1(1)	6(1)	1(1)
C(3)	22(1)	27(1)	23(1)	0(1)	4(1)	1(1)
C(4)	24(1)	25(1)	28(1)	-1(1)	0(1)	-2(1)
C(5)	21(1)	24(1)	32(1)	-1(1)	4(1)	-2(1)
C(6)	23(1)	37(1)	35(1)	-2(1)	0(1)	3(1)
C(7)	28(1)	44(1)	34(2)	-4(1)	-1(1)	-5(1)
C(8)	33(2)	58(2)	60(2)	8(1)	4(1)	-7(1)
C(9)	41(2)	79(2)	80(3)	10(2)	13(2)	-6(2)
C(10)	21(2)	84(3)	140(4)	-57(2)	20(2)	-18(2)
C(11)	46(2)	37(2)	73(2)	-2(1)	27(2)	-8(1)
C(12)	30(1)	42(1)	39(2)	6(1)	13(1)	-3(1)
C(13)	21(1)	31(1)	35(1)	-4(1)	8(1)	-1(1)
C(14)	22(1)	23(1)	30(1)	1(1)	7(1)	-2(1)
C(15)	24(1)	22(1)	26(1)	1(1)	6(1)	-1(1)
C(16)	25(1)	32(1)	30(1)	-2(1)	9(1)	-3(1)
C(17)	32(1)	37(1)	22(1)	1(1)	6(1)	-2(1)
C(18)	28(1)	29(1)	34(1)	4(1)	11(1)	5(1)
C(19)	22(1)	31(1)	34(1)	2(1)	4(1)	3(1)
C(20)	29(1)	25(1)	25(1)	0(1)	3(1)	4(1)
C(21)	37(1)	30(1)	29(1)	0(1)	8(1)	8(1)
C(22)	31(1)	23(1)	35(1)	-1(1)	11(1)	3(1)
C(23)	41(2)	34(1)	40(2)	0(1)	19(1)	2(1)
C(24)	46(2)	42(1)	36(2)	-4(1)	16(1)	6(1)
C(25)	49(2)	44(2)	45(2)	-3(1)	19(1)	4(1)
C(26)	52(2)	92(3)	67(2)	16(2)	26(2)	13(2)
C(27)	36(2)	85(2)	77(3)	-22(2)	18(2)	11(2)
C(28)	40(2)	39(1)	52(2)	2(1)	4(1)	15(1)

C(29)	30(1)	36(1)	40(2)	3(1)	0(1)	8(1)
C(30)	25(1)	30(1)	48(2)	0(1)	8(1)	3(1)
C(31)	26(1)	19(1)	38(1)	1(1)	8(1)	4(1)
C(32)	28(1)	21(1)	31(1)	0(1)	7(1)	5(1)
C(33)	28(1)	32(1)	31(1)	-1(1)	0(1)	4(1)
C(34)	37(1)	34(1)	27(1)	1(1)	5(1)	6(1)
C(101)	87(3)	76(3)	160(5)	-16(3)	59(3)	-3(2)
C(102)	86(3)	70(2)	93(3)	-2(2)	-1(2)	-4(2)
C(103)	82(3)	61(2)	69(3)	-3(2)	3(2)	-8(2)

Table 5 : Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for 1.

Atom	x	y	z	U(eq)
H(1A)	2300(30)	1737(16)	-75(18)	41(10)
H(1B)	3160(40)	1585(19)	515(19)	69(11)
H(2A)	3890(30)	3041(17)	3790(18)	47(10)
H(2B)	4010(30)	2882(15)	4554(18)	49(8)
H(2)	3115	3163	1974	33
H(6A)	9832	3099	3137	38
H(6B)	8968	2805	3711	38
H(7A)	8744	3909	4163	43
H(7B)	10309	3713	4180	43
H(8A)	8928	4613	3167	61
H(8B)	9733	4883	3885	61
H(9A)	11138	5111	3137	80
H(9B)	11751	4415	3492	80
H(10A)	11035	3789	2555	97
H(10B)	11768	4453	2294	97
H(11A)	8861	4537	2142	60
H(11B)	9853	4837	1626	60
H(12A)	10129	3718	1179	43
H(12B)	8565	3946	1061	43
H(13A)	8766	2780	1431	35
H(13B)	9730	3004	2125	35
H(16)	6913	3104	766	34
H(17)	4735	2987	162	37
H(19)	3179	1470	2520	35
H(23A)	9109	1484	1178	44
H(23B)	7908	1790	633	44
H(24A)	7367	697	175	48
H(24B)	8935	856	141	48
H(25A)	8431	-298	450	54
H(25B)	8042	-10	1170	54
H(26A)	10236	-559	1201	82
H(26B)	10717	113	823	82
H(27A)	10602	765	1749	78
H(27B)	11445	87	1995	78
H(28A)	8671	60	2209	53
H(28B)	9955	-254	2700	53
H(29A)	10582	860	3112	43
H(29B)	9090	664	3276	43
H(30A)	9137	1821	2883	41
H(30B)	9646	1572	2173	41
H(33)	7702	1490	3606	37
H(34)	5919	1590	4276	39
H(10C)	1124	4626	163	156

H(10D)	1851	5315	485	156
H(10E)	2265	4581	832	156
H(10F)	3179	4247	-189	101
H(10G)	2771	4983	-532	101
H(10H)	4718	4788	670	86
H(10I)	4308	5523	330	86